UNIVERSITY OF
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The Characterisation of Endometrial Epithelial Stem/Progenitor Cells

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by

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Abstract

The highly regenerative nature of the human endometrium has caused much speculation over the existence of resident stem/progenitor cells (SPCs) within this tissue. Endometrial SPCs may be associated with a variety of gynaecological pathologies such as endometriosis. Although common, little is known about the pathogenesis of this condition and the lack of defining markers of putative endometrial SPCs makes the isolation and analysis of these cells difficult. The well characterised endometrial stromal stem cells are only able to regenerate a stromal cell population of the endometrium in 3D *in vitro* or animal xenograft models. This suggests that there is a separate endometrial epithelial SPC that gives rise to the endometrial epithelial cells, including the glands and the surface epithelium. SSEA-1, a surface glycolipid expressed by embryonic stem cells (ESCs), has recently been identified as a possible endometrial epithelial progenitor cell marker by our lab. However, the gene expression profile of the SSEA-1$^+$ endometrial epithelial cells has yet to be assessed. With the use of qPCR, we aimed to further characterise the SSEA-1$^+$ endometrial epithelial cell population by assessing their gene expression profile for common markers of stemness and an undifferentiated state when grown in both 2D and 3D culture. As abnormal endometrial SPCs are speculated to be involved in the pathogenesis of endometriosis, we further investigated the gene profile of SSEA-1 expressing endometrial epithelial cells in this condition. Within the normal endometrium, we found no differences between the levels of stem cell markers expressed by the SSEA-1$^+$ and SSEA-1$^-$ epithelial cell populations grown in 2D culture. Significant up-regulation of markers of differentiation, *ERα* and *PR*, within the SSEA-1$^-$ cells confirmed the existence of a more differentiated cell state within this population. SSEA-1$^+$ epithelial cells grown in 2D culture exhibited significantly

higher levels of stem cell marker expression in patients with endometriosis than those with a normal endometrium, confirming their association with this disease. When grown in 3D culture endometrial epithelial cells form gland-like structures also called spheroids/organoids, similar to those seen within the endometrium. 3D Matrigel culture mimics the endometrium and the stem cell niche *in vivo* and therefore acts as a better culture system to preserve stemness and accurately reflects *in vivo* physiology. Unlike the 2D culture, SSEA-1$^+$ epithelial cells grown in 3D culture exhibited a clear up-regulation of markers of stemness when compared to the SSEA-1$^-$ cell population, in both normal and endometriosis tissue. This difference was more pronounced in cells taken from women with endometriosis, again indicating its link with this condition. High levels of *ERα* and stable levels of *PR* expression indicated elements of oestrogen responsiveness and progesterone resistance within the 3D cultured SSEA-1$^+$ epithelial cells taken from women with endometriosis, and are known features of endometriosis. These findings provide further evidence to suggest that endometrial epithelial SPCs are contained within the SSEA-1$^+$ cell population displaying greater stem cell activity than the SSEA-1$^-$ population, but only when grown in 3D culture which mimicks their *in vivo* environment. This highlighted the significance of the surrounding stem cell niche in preserving stemness and preventing differentiation. Furthermore, our study demonstrats that this sub-population of SSEA-1$^+$ epithelial SPCs are in some way involved in the pathogenesis of endometriosis.

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Glossary

5S rRNA. *5S ribosomal RNA*

18S rRNA. *18S ribosomal RNA*

28S rRNA. *28S ribosomal RNA*

ACTB. Beta Actin

ASCs. Adult Stem Cells

ASRM. American Society for Reproductive Medicine

BLAST. Basic Local Alignment Search Tool

BMDCs. Bone Marrow-Derived Stem Cells

BMI. Body Mass Index

BrdU. 5-Bromo-2-DeoxyUridine

BSA. Bovine Serum Albumin

CA 125. Cancer Antigen 125

CK. Cytokeratin

CSC. Cancer Stem Cell

DMEM/F12. Dulbecco's Modified Eagle Medium/F12

DNase. Deoxyribonuclease

DTT. Dithiothreitol

ECCs. Embryonic Cancer Cells

ECM. Extracellular Matrix

EDTA. EthyleneDiamineTetraacetic Acid

EGF. Epithelial Growth Factor

ER. Oestrogen Receptor

ERα. Oestrogen Receptor Alpha

ERβ. Oestrogen Receptor Beta

ESCs. Embryonic Stem Cells

FACs. Flow Cytometric Analysis

FBS. Fetal Bovine Serum

FC. Flow Cytometry

FISH. Fluorescence *in situ* Hybridisation

FITC. Fluorescein Isothiocyanate

FSH. Follicle-Stimulating Hormone

FucT. Fucosyltransferase

GAPDH. Glyceraldehyde-3-Phosphate Dehydrogenase

GCP. Good Clinical Practice

GnRH. Gonadotrophin-Releasing Hormone

hCG. Human Chorionic Gonadotrophin

HPO. Hypothalamo-Pituitary-Ovarian

HSC. Haematopoeitic Stem Cell

hTERT. Human Telomerase Reverse Transcriptase

hTR. Human Telomerase RNA

IF. ImmunoFluorescence

ITS. Insulin Transferrin Selenite

IHC. Immunohistochemistry

IQR. Interquartile Range

LMP. Last Menstrual Period

LRC. Label-Retaining Cell

LREC. Liverpool Research Ethics Committee

MACS. Magnetic Activated Cell Sorting

MMPs. Matrix Metalloproteases

MSC. Mesenchymal Stem Cell

MWU test. The Mann-Whitney U-test

NANOG. Nanog Homeobox

NCBI. National Center for Biotechnology Information

NGS. Normal Goat Serum

NSAIDs. Non-Steroidal Anti-Inflammatory Drugs

NTC. No Template Control

OCP. Oral Contraceptive Pill

PODXL. Podocalyxin

PR. Progesterone Receptor

qPCR. Quantitative Polymerase Chain Reaction

RT-PCR. Reverse Transcriptase Polymerase Chain Reaction

SPC. Stem/Progenitor Cell

TAE. Tris-Acetate-Ethylenediamine Tetraacetic Acid

TP1. Telomerase Protein 1

TRAP. Telomere Repeat Amplification Protocol

uNK. Uterine Natural Killer

VEGF. Vascular Endothelial Growth Factor

WSR test. Wilcoxon signed-rank test

YWHAZ. Tyrosine 3-monooxygenase

Chapter 1

Introduction

Chapter 1: Introduction

The human endometrium is a highly dynamic tissue, undergoing over 400 cycles of shedding and regeneration throughout a female's reproductive years. Due to its immense regenerative capacity, it has long been proposed that a putative adult stem cell (ASC) population must be driving its re-growth, yet it is only over the last 8 years since the first evidence emerged that research has been dedicated to the identification of such cells. The use of endometrial ASCs in regenerative medicine is an exciting prospect, acting as an easily accessible and renewable source. Endometrial ASCs are also said to be associated with the pathogenesis of a variety of gynaecological conditions such as endometriosis, about which little is known and treatment is still limited. As a result of previous studies, it is now established that two distinct stem cell populations give rise to the two main cell types of the endometrium, the stroma and the epithelium. To date, most research has focused on the stromal stem cell due to their ease of culture, yet very little is known about the epithelial cell population. It is therefore the main focus of this study, to help identify and characterise a putative epithelial stem/progenitor cell (SPC) within the endometrium. Before beginning this study, a thorough literature review of the scientific advances within this field had to be conducted.

1.1 Structure of the Human Uterus and Endometrium

The female reproductive system can be divided into two sections: the lower and the upper genital tracts. The lower genital tract consists of the vagina and vulva, whereas the upper portion is made up of the anatomical structures known as the uterus and cervix, along with the attached fallopian tubes and ovaries. The uterus is a thick,

muscular organ situated in the midline of the pelvic cavity between the urinary bladder and rectum (Sandring, 2008). It acts as the body of the genital tract, and is vital for hosting a pregnancy following the fertilisation of an ovum after its release from the ovaries and migration through the fallopian tubes. (Aplin, 2008). The uterus measures 7.5 cm in length and sits in an anterior-posterior position with its apex directed downwards and backward (Gray's Anatomy of the Human Body, 2012). The uterine body forms the upper two-thirds and tapers down to form a narrow neck known as the cervix, which in turn sits at the superior part of the vagina. The uppermost part of
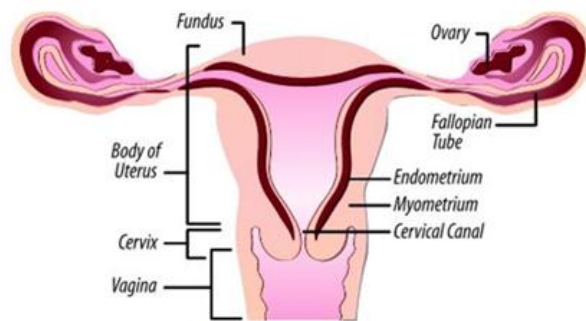
Figure 1.1 The human female reproductive system (NUFF, 2012)

the body is named the fundus. Although the morphology of the uterus varies amongst females of different species, its basic structure is common to all mammals (Aplin, 2008). The structure of the uterus is illustrated in the diagram shown (figure 1.1)(NUFF, 2012).

The uterine mucosa, also known as the endometrium, is the innermost layer lining the uterine cavity and plays an essential role in fetal development (Aplin, 2008). It is a highly dynamic and regenerative tissue found on the thick smooth muscle of the myometrium. These two tissue types meet at the irregular endometrial-myometrial junction with no submucosal tissue to separate them. Embryologically the endometrium and subendometrium are said to originate from the two paramesonephric ducts known as the Müllerian ducts, unlike the outer myometrium which later develops

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during fetal life (Gargett, 2007). Microscopically the human endometrium is composed of two main cell types: the epithelial cells which line the lumen and glands, and the supporting stromal or mesenchymal cells. Other cell types found within the endometrium include endothelial cells and leukocytes. The endometrium is divided into two separate layers, the functionalis and the basalis, based on their structural and functional differences. The functionalis layer, the upper two-thirds of the endometrium, is composed of dense glandular tissue surrounded by a loose connective stroma (Figueria *et al*, 2011). Its role is to accommodate an implanting blastocyst, and also to provide the maternal element of the placenta (Aplin, 2008). It is this layer which is lost during menses. On the contrary, the lower basalis is maintained following each menstrual cycle, and is known to contain the gland bases, dense stroma and large vessels. It is therefore thought that this basalis layer is responsible for the generation of a new functionalis each month and is where the endometrial SPCs must reside (Figueria *et al*, 2011). The histology of the human endometrium is shown in figure 1.2 (Gargett, 2007).
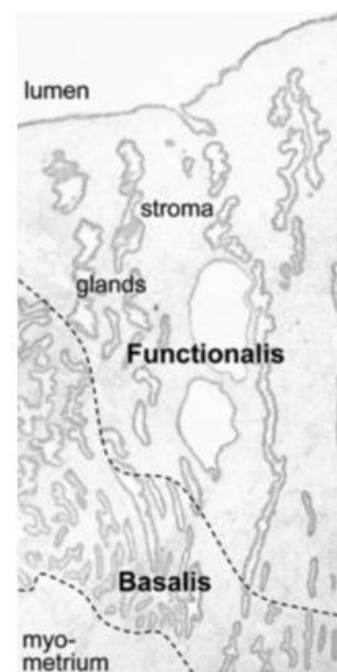
Figure 1.2 Histology of the human endometrium (Gargett, 2007)

1.2 The Human Menstrual and Ovarian Cycles

The uterus and its endometrial lining play an essential role in the key events of primate reproduction, implantation and when in the absence of pregnancy, menstruation (Critchley *et al*, 2006). During a woman's reproductive years, the endometrium

undergoes over 400 cycles of monthly regeneration, differentiation and shedding in response to hormonal changes that occur within the body (Gargett *et al*, 2010). This dynamic remodelling and cyclical change ultimately occurs to provide the optimal environment for embryo implantation and is tightly controlled by endocrine, autocrine and paracrine factors that not only influence the endometrium, but follicle maturation and ovulation also. The transformation of the endometrium throughout the cycle comes with classical morphological and histological changes, which allow for dating the cycle with the use of endometrial biopsies. It is important to note that whilst many of the fundamental reproductive processes are common between different mammalian species, the mechanism for implantation varies widely and therefore to complement this endometrial preparation and remodelling differ also (Aplin, 2008; Mihm *et al*, 2011).

1.2.1 The Human Menstrual Cycle

Women first start menstruating during the final stage of puberty, also known as menarche, commonly between the ages of 8.5 to 13 years. A woman's reproductive years span around 36 years, ending when she reaches the menopause around the age of 51 (Mihm, 2011). For most women, the standard cycle length is 28 days, although this commonly fluctuates between 26 to 32 days. The new cycle begins with the first day of menstrual bleeding, with ovulation occurring mid-cycle around day 14. The menstrual cycle in all women can be described by dividing it into three phases; menstrual, proliferative (or follicular) and secretory (or luteal). Broadly speaking, menses encompasses the breakdown of the functional endometrium, although simultaneous re-epithelialisation is also initiated during this phase. Following this, there is rapid

cellular proliferation and generation of a new extracellular matrix (ECM) in response to the secretion of oestrogen; hence this phase is named the proliferative phase (Aplin, 2008). During the process of endometrial regeneration, the mucosal tissue can grow up to 8mm in thickness (Kyo *et al*, 2011). Following ovulation, within the secretory phase of the cycle progesterone stimulates functional differentiation of the endometrium in preparation for implantation. Endometrial glands become increasingly active and more tortuous and following day 22 stromal differentiation and endometrial dicidualisation favour successful implantation and placentation. This short period of heightened uterine receptivity towards embryo implantation within this phase is named the 'window of implantation'. If fertilisation does not occur, hormone levels subsequently fall, the endometrium begins to regress and the cycle starts over again (Aplin, 2008; Buffet *et al*, 1998). A schematic of the human menstrual cycle is illustrated in figure 1.3 (Gargett *et al*, 2008).
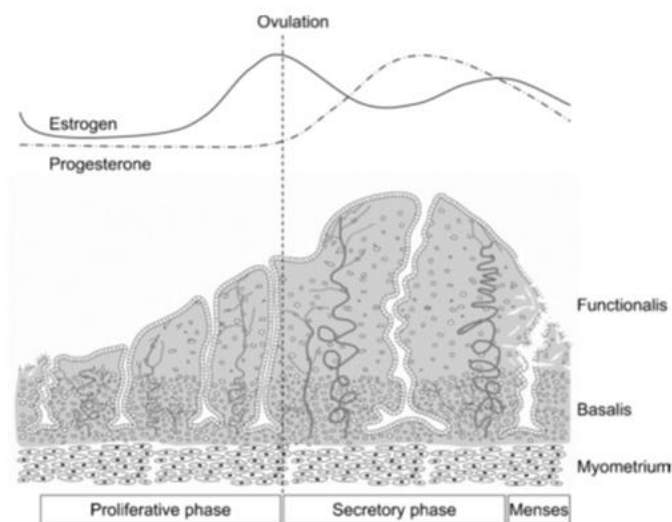
Figure 1.3 Diagram illustrating the phases of the human menstrual cycle under hormonal influence (Gargett *et al*, 2008)

Menses

If implantation and pregnancy do not occur, a demise of the corpus luteum leads to the rapid drop in levels of the ovarian steroid hormones, progesterone and oestrogen, and initiates endometrial breakdown between days 1 to 4 of the cycle. Spiral arteries within the functionalis undergo repetitive constriction and relaxation, eventually leading to a loss of vascular integrity. Bleeding into the stromal compartment of the endometrium causes cleavage between the basal and functional layers, the latter of which is completely lost during menstruation. This eventually leads to tissue apoptosis and necrosis, a loss of cellular adhesion and cell membrane actin, and the breakdown of the glandular epithelium. In addition, endothelial injury promotes the release of inflammatory mediators such as prostaglandin F2α and E2, platelet aggregation and the formation of thrombin within the basal layer. Rising levels of prostaglandins in turn stimulate myometrial contractility and vasoconstriction of the spiral arteries. Local responses are mediated by leucocytes which secrete matrix metalloproteases (MMPs). These MMPs cause direct destruction of the cellular membranes and a loss of the ECM. Towards the end of menses, on day 3, haemostasis occurs within the basal layer and surface re-epithelialisation and regeneration occurs under the control of oestradiol, originating from the glandular stumps. Menstrual bleeding is an external manifestation of the internal physiological processes which occur during menses, with the heaviest flow usually noticed at day 2 (Aplin, 2008; Mihm *et al*, 2011).

Proliferative Phase

The proliferative phase occurs between days 5 to 14, up to the time of ovulation. Under the influence of oestrogen, throughout this phase there is extensive growth and re-

epithelialisation of the endometrium and it increases in thickness from 1mm up to 3-4mm. Early repair of the endometrium occurs whilst oestrogen levels are low and epithelial cells do not express oestrogen receptor α (*ERα*). During this stage, oestrogen levels rise and both the epithelial and stromal cells gain the surface receptors *ERα* and progesterone receptor (*PR*). Under the control of these hormones, throughout days 5-7 (early proliferative phase) the functionalis is regenerated and there is vast proliferation of the glandular epithelium and to a lesser degree, the stroma. Histologically the cellular morphology changes throughout the proliferative phase. Within the early proliferative phase (days 5-7), endometrial glands are undifferentiated and straight in appearance. Under cross section the glands look circular and are seen to be lined with columnar epithelial cells containing nuclei near the basal surfaces. The glandular epithelium stays relatively constant in size throughout this period, and occasionally cells can be seen undergoing mitosis. Stromal cells appear spindle-like in shape and contain large nuclei. As this phase progresses to between days 8 and 10 (mid-proliferative phase), the endometrial glands appear taller and more tortuous. Towards days 11 to 14 (late proliferative phase), the glands becomes increasingly pseudostratified and tortuous in morphology. Mitotic figures become more pronounced and stromal oedema becomes apparent (Aplin, 2008; Gargett *et al*, 2008).

Secretory Phase

After ovulation at day 14, the secretory phase begins and extends up to day 28. This part of the menstrual cycle is commonly separated in to three parts: the early, mid and late secretory phases. It is during this phase that all endometrial cell types undergo function differentiation, priming the endometrium for the implantation of an embryo.

Proliferation is suppressed and under the influence of progesterone produced by the corpus luteum, differentiation begins. As the name suggests, the endometrium produces large amounts of secretions during this phase, named histotroph. Although the exact composition of these secretions is still unknown, it is said to contain transport proteins, glucose, hormones, growth factors and enzymes in addition to other substances. In order to prepare for blastocyst implantation, the glandular epithelia begin to secrete large amounts of glycogen and histotrophic secretory products under the influence of progesterone. Progesterone also triggers the influx of specialised uterine natural killer (uNK) cells as a response to the release of local chemokines. uNK cells are an important source of both growth and angiogenic factors, and they enable spiral artery remodelling required in preparation for a pregnancy. During the majority of the menstrual cycle, the endometrium is named as either 'hostile' or 'non-receptive' to an incoming blastocyst, except for a short period during the mid-secretory phase named the 'window of receptivity/implantation'. Progesterone inhibits the action of ERα and epithelial PR within the functionalis, although they remain functional within the basalis. Within the stromal compartment of the functional layer, PR remains active and these cells differentiate and undergo pre-decidual changes. In response to this postovulatory increase in progesterone, endometrial stromal cells are seen to change greatly in morphology, forming secretory epithelioid-like decidual cells (Aplin, 2008; Gargett *et al*, 2008; Gellerson *et al*, 2007).

1.2.2 The Ovarian Cycle

Synchronised with the cyclical changes of the endometrium, the ovaries go through their own physiological changes. Analogous to the male testes, the ovaries are the

reproductive organs within females and act as both gonads and endocrine glands. Under the influence of the hypothalamo-pituitary-ovarian (HPO) axis, the ovaries control follicular maturation in the aim to produce one mature oocyte each month. Gonadotrophin-releasing hormone (GnRH) is released from the hypothalamus in a pulsatile manner which in turn stimulates the pituitary gland to secrete luteinising hormone (LH) and follicle-stimulating hormone (FSH). The ovaries respond to the release of FSH and LH in a cyclical manner called the ovarian cycle and in turn secrete the ovarian hormones oestrogen and progesterone (Buffet *et al*, 1998; Sadler *et al*, 2006). Primordial germ cells (PGSs) are diploid precursors of female and male gametes. In order to develop into an egg ready for ovulation, it must pass through several stages of maturation through a process called oogenesis. PGSs arise in a developing embryo at around 3 weeks post-fertilisation. These exist only transiently during which they multiply and migrate to the genital ridge, where they are then named oogonia. The population of oogonia continue to proliferate and expand via mitosis until they stop and enter meiosis. At this point they are now called primary oocytes. Primary oocytes remain arrested in prophase I of meiosis until the female reaches sexual maturity during puberty. The ovaries of a female embryo contain 7 million oocytes *in utero*. Of these, only around 400 ovulate and the remainder undergo atresia. The next stage in oocyte development continues when meiosis is resumed in preparation for ovulation. It then forms a secondary oocyte and in addition releases the first polar body. The secondary oocyte is arrested at meiosis II until fertilisation occurs and a haploid gamete is produced.

Primordial follicles are the basic functional units of the ovary and contain the developing oocyte. Like the ooctye, an ovarian follicle must also grow and mature before ovulation can occur, in a process called folliculogenesis. Entry of the oogonia into meiosis I initiates the development of the primordial follicle. As this primordial follicle grows it must pass through a series of stages through which it becomes a primary follicle, a secondary follicle and finally a tertiary or pre-ovulatory follicle. A primordial follicle is made up of a primary oocyte surrounded by a layer of flattened granulosa cells. During the development of primary follicles into tertiary follicles, the granulosa cells proliferate and undergo changes from a flattened to a cuboidal epithelial morphology. Eventually this process gives rise to a follicle with multiple granulosa cell layers surrounded by an outer layer consisting of thecal cells and a basement membrane (Smitz *et al*, 2002; Mtango *et al*, 2008).

Like the menstrual cycle, the ovarian cycle too lasts 28 days. It is split into two phases, the follicular and luteal phase, each lasting 14 days (Buffet *et al*, 1998).

Follicular Phase

At puberty, the levels of circulating FSH rise and a cohort of mature follicles are selected for initial recruitment. From these, a single dominant follicle is chosen. It takes 150 days for a primordial follicle to mature into a primary follicle and a further 120 days to develop into a secondary follicle. This means that it takes the equivalent of 9 menstrual cycles until a follicle can become part of a selectable cohort. The 15 days following this is the time taken for a selected follicle to become a dominant and equates to the period of the follicular phase. Nearing the end of the menstrual cycle, if

a pregnancy is not achieved, the corpus luteum starts to regress and the levels of oestrogen and progesterone begin to fall. These decreasing hormone levels stimulate the anterior pituitary gland to increase the secretion of FSH, which in turn stimulates a cohort of follicles to grow. Of these, one follicle will grow at the faster rate than the others and will secrete higher levels of oestradiol (E2) and inibin-B. These hormones, produced by the dominant follicle, have a negative effect on the pituitary and cause suppression of FSH secretion around the mid-follicular phase. This withdrawal of FSH results in apoptosis of the remaining non-dominant follicles. FSH activates LH receptors within the theca cells of the dominant follicle and in turn stimulates its production of E2. E2 has a stimulatory effect on FSH and LH which surge around day 14 and result in the meiotic maturation of the oocyte and follicular rupture to release the ovum in a process known as ovulation. The tissue that remains behind forms a body known as the corpus luteum (Tulsiani, 2003).

Luteal Phase

The surge of LH around the time of ovulation causes the corpus luteum to become leutinised and synthesise oestrogen and more importantly progesterone. The corpus luteum stays functional for a period of 14 days, under the control of LH. In the absence of a pregnancy, the corpus luteum begins to degenerate and is replaced by connective tissue termed the corpus albicans. In the event that fertilisation does occur, the developing blastocyst secretes human chorionic gonadotrophin (hCG) which prevents breakdown of the corpus luteum. During the final 4-5 days of the cycle plasma levels of oestrogen and progesterone decline rapidly, previous suppression of GnRH and FSH is removed and the cycle repeats itself (Tulsiani, 2003).

1.3 Stem Cells

1.3.1 Definition of Stem Cells

Stem cell biology and its application in future regenerative medicine, is currently one of the most exciting and continually expanding areas of biomedical research (Sylvester *et al*, 2004). The concept of a stem cell is difficult to define and has been the cause of much controversy over the years. Their lack of morphological features makes them difficult to identify and locate within tissues, and so alternatively they are described by their characteristic functional properties (Potten *et al*, 1990; McCullock *et al*, 2005). Within the literature, stem cells are commonly defined as a rare population of undifferentiated cells which are able to divide in order to maintain their pool within the tissue and also to give rise to specialised tissue-specific cells with defined functions. In order for a cell to possess the characteristics of a stem cell, it must possess three functional properties: a high proliferation potential, the ability to self renew and the capacity to differentiate (Gargett, 2007). The proliferation potential of a cell is determined by calculating the number of times a cell population doubles from the existence of a single cell until senescence (Gargett, 2007; Cervello *et al*, 2011). Self renewal is known as the ability of a cell to divide producing an identical daughter cell, ultimately allowing it to retain its population within the tissue (Bach *et al*, 2000). Differentiation on the other hand is defined as the change of cell phenotype based on gene expression associated with cell function rather than cell division (Figueira *et al*, 2011).

1.3.2 Stem Cell Hierarchy

Stem cells display a varying ability to differentiate, depending on their position within the hierarchy of stem cells. This is illustrated in figure 1.4 (Kyo *et al*, 2011).
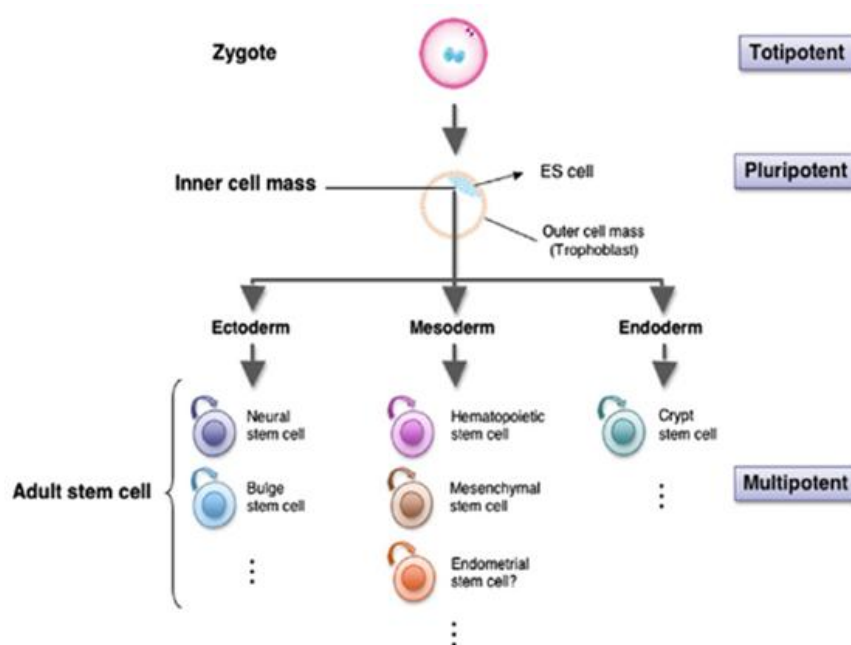
Figure 1.4 Hierarchy of stem cells (Kyo *et al*, 2011)

'Totipotent' stem cells, such as the zygote, are fully undifferentiated cells positioned at the top of this hierarchy. These have the ability to produce cells of all three embryonic germ layers (endoderm, mesoderm and ectoderm), in addition to cells derived from extra-embryonic tissues (these being the trophoblast, placenta and extra-embryonic membranes) (Figueira *et al*, 2011). Next in line are embryonic stem cells (ESCs), derived from the inner cell mass of the blastocyst. These are named 'pluripotent' as they can give rise to an embryo, and were first isolated in mice in the year 1981 and in humans in the late 1990s. They are not able to produce extra-embryonic tissues (Kyo *et al*, 2011; Eckfeldt *et al*, 2005). As embryonic development continues, the differentiation potential of these cells becomes ever more restricted. ASCs, also referred to as somatic stem cells (SSCs), arise from these and are found in most adult

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tissues. They can be classified as either 'multipotent' or 'unipotent', dependent on whether they can produce multiple cell types in a germ cell lineage or into only one cell type within that lineage respectively (Figueira *et al*, 2011; Kyo *et al*, 2011; Aplin, 2008).

ASCs in different tissues, such as haematopoietic, neural, epidermal and even gastrointestinal stem cells, have now been identified. They act to maintain tissue homeostasis by generating and regenerating adult tissue during the processes of normal physiological cell turnover and in the event that tissue damage may occur. However they exhibit a much lower self-renewal capacity when compared to that of ESCs (Eckfeldt *et al*, 2005; Gargett *et al*, 2010). To date, the best described ASCs are those derived from the bone marrow, including haematopoietic and mesenchymal (or stromal) stem cells (MSCs) (Cervello *et al*, 2011). The particular topic surrounding ASC plasticity and therefore the fate of ASCs, has caused much controversy within the literature. Contrary to common belief, it is now suggested that ASC can undergo transdifferentiation or plasticity, in which the cells can be converted from one cell lineage to another as a result of a change in the extracellular environment. This property has been described within the stem cells of the bone marrow. They are seen to travel within the blood stream and are eventually incorporated for use in the regeneration of damaged tissues such as muscle, the brain, heart or the liver. The implications of such research findings are important as it may imply that ASCs are more similar to ESCs than originally thought, and could have alternative therapeutic uses. Due to the rarity of stem cell plasticity however, this concept if often rejected and alternative explanations given (Gargett, 2007; Wagers *et al*, 2004).

1.3.3 Stem Cell Division

Stem cells demonstrate two different mechanisms of cellular division which enable the stem cell pool to be maintained. These are named asymmetric and symmetric cell division. Asymmetric division (shown in figure 1.5 (Gargett, 2007)) is demonstrated when a stem cell produces an identical
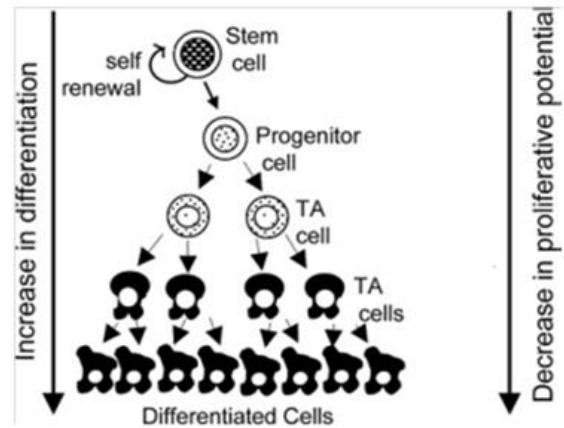
Figure 1.5 Asymmetric cell division (Gargett, 2007)

daughter cell, thereby exhibiting the property of self-renewal. Simultaneously a more differentiated cell, also known as a progenitor cell is produced (Aplin, 2008). Progenitor cells are tissue-specific cells which are set on a particular path of differentiation and therefore only have a limited ability to self-renewal. These progenitor cells in turn give rise to transient amplifying (TA) cells, which have properties mid-way between stem cells and fully differentiated mature cells. TA cells proliferate rapidly, progressively gaining in differentiation markers and eventually produce terminally differentiated cells (Gargett, 2007; Kyo *et al*, 2011). In contrast, a stem cell may alternatively choose to undergo symmetric cell division, producing either two daughter stem cells, or two TA progenitor cells (Aplin, 2008).

1.3.4 The Stem Cell Niche

Stem cells reside within and are regulated by a special physiological microenvironment that is tailored to its needs, as first described by Schofield in 1978. This is commonly referred to as the stem cell niche (Schofield, 1978). The structure and location of the

stem cell niche is variable depending on the resident tissue. Much of what is known about the stem cell niche has been discovered due to the work on other organisms, such as the *Drosophila melanogastor* and *Caenorhabditis elegans* gonads. Although less is known in comparison about the more complex mammalian stem cell niches, similarities can be drawn between the two (Ohlstein *et al*, 2004). ASCs are found to have a precise location within the niche, and are surrounded by mature, fully differentiated cells in an ECM. Adhesion molecules, such as cadherins and integrins, and the surrounding niche cells aid in anchoring the ASCs during periods of inactivity and manage the controlled release of daughter cells from this micro-environment following asymmetrical cell division. The essential function of this niche is to regulate signals controlling the balance between ASC self renewal for tissue replacement and cell differentiation, thereby preserving homeostasis within the tissue (Gargett, 2007; Cervello *et al*, 2011; Li *et al*, 2005).

1.4 Stem Cells and the Endometrium

As previously discussed, the endometrium is a highly dynamic and regenerative tissue undergoing monthly cycles of shedding and regeneration throughout a woman's reproductive years. In addition to this cyclical renewal, physiological regeneration is observed within the endometrium following childbirth, post-ablative therapy, post-curettage and also within postmenopausal women given oestrogen replacement therapy (Gargett CE, 2008). This high cellular turnover is comparative with other organs in which a high turnover rate is also required, including the haematopoietic system, the epidermis and the intestinal epithelium in which stem cells have also been located (Cervello *et al*, 2011; Aplin, 2008). It is this combination of reasons that has caused

many to believe that stem cells may be responsible for endometrial regeneration. It was first postulated in 1978 by Prianishnikov and later by Padykula in 1984, that stem cells located within the basalis may be the drive behind the endometrium's impressive regenerative capacity (Prianishnikov, 1978; Padykula *et al*, 1984).

1.4.1 Human Endometrial Stem/Progenitor Cells: The Evidence

Indirect Evidence

Indirect evidence for the presence of endometrial ASCs has gathered over the years. Early kinetic studies investigating endometrial cell proliferation showed zonal differences determining the orderly replacement of the endometrial epithelial and stromal cells from rarely proliferating SPCs within the basalis (Ferenczy *et al*, 1979; Conti *et al*, 1984; Padykula *et al*, 1989). Later in 2003, Brenner *et al* observed a difference in the proliferative index between the glands of the functionalis and basalis layers, across both the proliferative and secretory stages of the menstrual cycle (Brenner *et al*, 2003). In 2005 endometrial epithelial stem cell kinetics were explored, by investigating epigenetic errors that had been encoded into particular methylation patterns of individual endometrial glands. The purpose was to determine the total number of stem cell divisions by calculating the number of somatic errors that would accumulate within a gland. An age-related increase in methylation was found up until the menopause, when methylation became constant, and therefore was seen as a reflection of stem cell mitosis (Kim *et al*, 2005). More recently, evidence emerged reporting the monoclonality of the endometrial glands, as proof that they arise from a single SPC. Within the normal proliferative endometrium, a rare population of glands

found unable to express phosphatase and tensin (PTEN null glands), are seen to regenerate their mutant glands with each cycle (Mutter *et al*, 2000).

These indirect findings have also been supported by various clinical observations suggesting further evidence to pursue the stem cell hypothesis. Complete endometrial regeneration which is able to support a pregnancy following almost complete resection by curettage, has been seen in the clinical setting and also in primate studies (Hartman, 1993; Wood *et al*,1993). In addition, this regeneration can be seen within the endometrium of women treated for menorrhagia with electrosurgical ablative therapy (Tresserra *et al*, 1999). The endometrium's incredible ability to undergo ossification following trauma, such as a termination of a pregnancy, is said to be due to the incorporation of MSCs into the regenerating tissue (Biervliet *et al*, 2004; van Os *et al*, 2004). Other tissue types such as smooth muscle, bone and cartilage have also been found in the uterine lining and suggest an ability to differentiate (Bird *et al*, 1965; Roth *et al*, 1966; Mazur *et al*, 1980).

Evidence from Human Studies

Although many had suspected the existence of resident SPCs within the endometrium for some time, it was only until much more recently that direct evidence based on functional assays was published. In 2004, Chan *et al* used the concept of cell cloning to demonstrate the presence of separate epithelial and stromal stem cells in the human endometrium. Following single cell suspensions, $0.22 \pm 0.07\%$ and $1.25 \pm 0.18\%$ of epithelial and stromal cells formed individual colonies after 15 days, with large and small colonies formed from both cell types. Chan *et al* postulated that the large

colonies were formed from the resident SPCs with a greater ability to self-renew, whereas the small colonies were probably initiated by TA cells (Chan *et al*, 2004). In 2005, Schwab *et al* provided further evidence that the proportions of clonogenic epithelial and stromal cells do not change across the stages of the menstrual cycle or in the inactive endometrium, supporting the hypothesis that putative endometrial SPCs must be located within the basalis (Schwab *et al*, 2005) (see figure 1.6 (Gargett, 2007)).
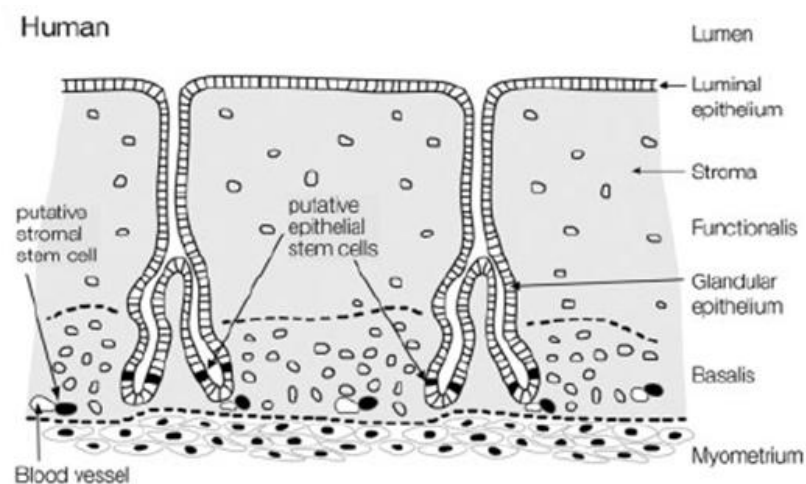
Figure 1.6 Hypothesised location of putative stem/progenitor cells within the endometrium (Gargett, 2007)

Stem cell subpopulations have been identified and analysed within previous studies using the 'side-population' approach, which is universally recognised as a marker of ASC activity (Challen *et al*, 2006). Side population (SP) cells, are a small number of cells which have the ability to efflux the fluorescent DNA-binding dye Hoechst 33342 more rapidly, when compared to other cell populations. This occurs because SP cells are said to have an increased level of ABCG2/Bcrp I expression, an ATP-binding plasma membrane transporter protein, allowing the characteristic SP phenotype to be detected using flow cytometry (FC) (Kyo *et al*, 2011; Gargett *et al*, 2007). Several studies have all demonstrated the presence of SP cells within freshly isolated and also

short term cultures of human endometrial cells (Tsuji *et al*, 2008; Cervello *et al*, 2009; Masuda *et al*, 2010; Kato *et al*, 2007). Kato *et al* found that the percentage of SP cells varied greatly between subjects, from 0 to 5.11%. SP cells have been found in highest proportion within the proliferative and menstrual stages on the menstrual cycle (Kato *et al*, 2007; Tsuji *et al*, 2008). The majority of the SP cells sorted from short-term cultures were found to be negative for both endometrial epithelial (CD9) and stromal (CD13) cell differentiation markers, however in long term Matrigel culture the aggregates demonstrated the capacity to form $CD9^+E$-cadherin$^+$ gland like-structures and also $CD13^+$ stromal-like clusters (Kato *et al*, 2007). SP cells cultured on feeder cells, showed slow proliferation and a colony forming ability comparable with the characteristics of SPCs, and were kept alive for over 9 months. This contrasts with the non-SP cells which became senescent following only 3 months (Masuda *et al*, 2010).

Studies exploring the important functional stem cell property of differentiation and multi-potency have emerged recently within the human endometrium, however so far this has only been investigated within the endometrial stromal cell population. It has been documented that a subset of these stromal cells, taken from women within their reproductive years, could differentiate into adipogenic, osteogenic, myogenic and chondrogenic mesodermal cell lineages when cultured in the appropriate induction media (Schwab *et al*, 2007; Gargett *et al*, 2009; Dimitrov *et al*, 2008). Wolf *et al* demonstrated that amongst tissue biopsies taken from the endometrium, myometrium, fallopian tubes and uterosacral ligament, only cells taken from the endometrium were able to undergo chondrogenic differentiation following 21 days of culture (Wolf *et al*, 2010). In the same study that proved epithelial cell clonogenicity, Gargett *et al*

continued to demonstrate that these same cells form spheroid structures positive for the epithelial marker cytokeratin in 3D Matrigel culture (Gargett *et al*, 2009). Kato *et al* also confirmed these findings by showing that epithelial SP cells aggregate into gland-like structures in Matrigel, expressing the epithelial markers CD9 and E-cadherin following 5 months of culture. Stromal SP cells did not behave in the same way (Kato et al, 2007).

Although evidence is available to propose the differentiation ability of the endometrium, *in vivo* studies by Masuda *et al* and Cervello *et al* suggest otherwise. If an ASC is multi-potent and therefore has the ability to differentiate, in theory it should be able to adapt when placed in a different environment, giving rise to cells of the new tissue type (provided that the tissue has the same embryological origin). The kidney, like the endometrium originates from the intermediate mesoderm. However, when transplanted under the kidney capsule of immunocompromised mice, endometrial SP cells (epithelial and stromal mix) and pure stromal SP cells gave rise to endometrial tissue containing glandular and stromal structures at the site of transplantation. This raises some question as to the endometriums ability to differentiate (Masuda *et al*, 2010; Cervello *et al*, 2010).

Evidence from Mouse Studies

The label-retaining cell (LRC) technique, like the SP phenotype, is another method used to identify and locate adult endometrial stem cells within the niche (Chan *et al*, 2006; Cervello *et al*, 2007; Szotek *et al*, 2007; Kaitu'u-Lino *et al*, 2010). Unfortunately due to the nature of this technique however, LRCs cannot be used in humans.

Although mice do not menstruate, but rather undergo cycles of proliferation and apoptosis, the mouse is still a well established animal model used to investigate the human endometrium due to its structural similarity (Aplin, 2008). The LRC approach uses the thymidine analogue, 5-bromo-2-deoxyuridine (BrdU), which once administered is taken up by the DNA of growing cells and therefore is commonly used for cell proliferation assays. Once labelled and the cells begin to divide, the label gets diluted and over time is eventually lost. Based on the principle that SPCs are slowly dividing and quiescent in nature, they retain the BrdU label over a long period of time and therefore can be detected even following many cell divisions (Kyo *et al*, 2011; Gargett CE *et al*, 2007). 6-9% of stromal cells, mainly located next to blood vessels near the endometrial-myometrial junction, were found to be LRCs. This corresponds to the hypothesised basal location of the SPCs within the endometrium (Chan *et al*, 2006; Cervello *et al*, 2007). Epithelial LRCs were found in 3% of the mouse endometrial epithelial cell population, and were seen within the luminal epithelium rather than the basal glands. This could be explained by the fact that unlike humans, mouse endometrium is not lost through menstruation, and so may in fact be where the SPCs reside in mice (Chan *et al, 2006). Another study by Kaitu'u-Lino *et al* used the LRC technique on a mouse model mimicking the events of the human menstrual cycle. They identified that glandular epithelial cells were highly proliferative during the period of endometrial repair, but not before breakdown, as opposed to luminal epithelial cells which proliferated throughout both. The majority of the glandular epithelial LRCs were also found to be *ERα* positive during the time of repair. These findings suggest that only the glandular epithelium selectively proliferates during endometrial repair, in response to oestrogen. In addition, 7% of glandular epithelial LRCs were *ERα* negative

even throughout repair, and so may have located SPCs based on the theory that stem cells should remain in an undifferentiated state and therefore would be *ERα* negative (Kyo *et al*, 2011; Kaitu'u-Lino *et al*, 2010).

1.4.2 The Origin of Endometrial Stem/Progenitor Cells

Initially it was first postulated that foetal epithelial and MSCs persist in the adult uterus following embryonic development, and are responsible for the replenishment of the functional layer of the endometrium during each menstrual cycle (Snyder *et al*, 2005). Studies have now shown however, that there may also be an additional bone marrow-derived element. It is possible that bone marrow-derived stem cells (BMDCs), which have the capability of differentiating into both haematopoietic and MSCs, may migrate to damaged tissues such as the endometrium. They may transdifferentiate into cells of the damaged tissue and may also contribute to angiogenesis, allowing repair (Figueira *et al*, 2011). In 2004, Taylor *et al* performed reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC) on the endometrial biopsies of four women who received HLA-mistmatched bone marrow transplants from other female donors. The results showed 0.2-48% of chimerism in the epithelial cells and 0.3-52% in the stroma across the transplant recipients, indicating that BMDCs must have been involved in the re-growth of the endometrium. The extent of chimerism seemed to correlate with the length of time elapsed since the time of transplantation (Figueira *et al*, 2011; Kyo *et al*, 2011; Taylor, 2004). As further evidence, a follow-up study was conducted in mice, in which female recipients received bone marrow cells from male donors. 0.02% of endometrial glands and 0.03% of stromal cells were shown to be Y chromosome-positive with fluorescence *in situ* hybridisation (FISH)

analysis (Du *et al*, 2007). A similar human study by Ikoma *et al* using sex-mismatched donors, revealed that 0.6-48% of glandular epithelial cells and 8.2-9.8% of stromal cells were Y chromosome-positive on FISH analysis (Ikoma *et al*, 2009).

Although this evidence suggests that there must be a BMDC component to the regeneration of the endometrium, many unanswered questions still remain. More research needs to be undertaken to explore whether BMDCs normally migrate to the endometrium with each cycle, or whether this only occurs during transplantation. In addition, further research needs to investigate which type of BMDC is responsible for this (Gargett, 2007; Kyo *et al*, 2011).

1.5 The Identification and Characterisation of Endometrial SPCs

1.5.1 Stem Cell Markers

Due to the lack of specific surface markers and the general scarcity of ASCs within tissues, the identification and characterisation of these SPCs is often found difficult. Instead ASCs have been investigated in the literature by using stem cell assays, to explore the aforementioned key functional properties that a stem cell should possess. Haematopoeitic stem cells (HSCs) are the best characterised ASCs. In comparison to other ASCs, HSCs are relatively easy to analyse due to their non-adherent and isolated existence within the bloodstream. Therefore it is not surprising that these cells initially served as a model for stem-cell biology and that it was on these cells that *in vitro* and *in vivo* functional assays were first developed. Although many have adapted these surrogate assays to investigate stem cells within other tissues, it is proving difficult when attempting to apply them to adherent cells that are likely to behave differently

when taken out of their natural microenvironment, in this case the endometrium. A lot of research has been devoted to the identification of stem cell markers within somatic tissue. The detection of such markers would allow the prospective isolation of stem cells in order to enable further characterisation, localisation within the tissue and also their use within medical therapies. Studies evaluating potential markers need to ensure that these marker expressing cells also display the functional properties of SPCs, as many markers currently used are also found on mature cells and therefore their expression does not necessarily imply stem cell activity. CD34 for example is classed as a HSC marker, however it is also found of the surface of mature endothelial cells. Another difficulty with surface markers, is that cells change their phenotype in culture and therefore the marker expression profile *in vitro* may not represent the markers expressed when *in vivo* (Aplin, 2008).

1.5.2 Stem Cell Markers and the Endometrium

There are currently no specific surface markers for endometrial epithelial SPCs that allow them to be distinguished from their mature progeny, and therefore would enable their prospective isolation for further characterisation (Aplin, 2008). The discovery of a specific endometrial epithelial and stromal SPC marker has been the target of many studies, as this is fundamental for the characterisation of undifferentiated stem cells. Although certain markers have been shown to be expressed within the endometrium, none have been proven to be cellular specific to the epithelial stem cell population (Oliveiria *et al*, 2012).

Endometrial stromal stem cells have recently been characterised by the co-expression of two peri-vascular markers, CD146 and PDGF-receptor-β (PDGF-Rβ) and also the novel single marker W5C5 (Gargett CE, 2009; Masuda H, 2012). The CD146$^+$PDGF-Rβ$^+$ sorted stromal cells were found to be enriched 8 fold for colony forming units (CFU) when compared to the unsorted stromal population. This sorted subpopulation also proved to exhibit multi-potency when cultured under the correct induction conditions. (Gargett *et al*, 2009; Schwab *et al*, 2007; Cervello *et al*, 2011). W5C5$^+$ endometrial stromal cells make up $4.2 \pm 0.6\%$ of the total population of stromal cells within the tissue, and have also exhibited a significant clonogenetic and multi-lineage differentiation ability when compared to their W5C5$^-$ counterpart. W5C5$^+$ sorted stromal cells have also shown to give rise to stromal-like tissue *in vivo* (Masuda *et al*, 2012).

A number of other markers that have also been associated with the endometrium. Some of these studies have been summarised in table 1.1 (Oliveiria *et al*, 2012). The expression of the survival marker bcl-2, and the haematopoietic markers c-kit (CD117) and CD34 have been identified in the endometrium of hysterectomy tissues (Cho *et al*, 2004). Their importance as stem cell markers is questionable however, as they are seen to be expressed by a wide number of endometrial cells other than the clonogenic or SP endometrial cells identified in functional studies (Chan *et al*, 2004; Kato *et al*, 2007).

Table 1 Summary of stem cell markers in the human eutopic endometrium and in endometriotic lesions (Oliveiria *et al*, 2012)

	C-Kit		Oct-4		Notch-1		Musashi-1		Sox-2		Nanog	
	E.E.	E.L.	E.E.	E.L.	E.E.	E.L.	E.E.	E.L.	E.E.	E.L.	E.E.	E.L.
Osuga et al., 2000		+ PCR (P.F.)										
Elmore et al., 2001	+ IHC (P.E. and S.E.)											
Cho et al., 2004	+ IHC (P.E., S.E. and P.)											
Uzan et al., 2005	+ IHC	+ IHC										
Matthai et al., 2006			+ PCR and IHC									
Cobellis et al., 2008					+ IHC (P.E. and S.E.)							
Gotte et al., 2008					+ IHC	+ IHC	+ PCR and IHC	+ PCR and IHC				
Forte et al., 2009			+ PCR and IHC	+ PCR and IHC								
Gotte et al., 2010			+ PCR	+ PCR					+ PCR and IHC(P.E.>S.E).	+ PCR and IHC	+ PCR	+ PCR
Bentz et al., 2010			+ PCR and IHC									

E.E.: Eutopic endometrium; E.L.: Endometriotic lesion; P.F.: Peritoneal fluid; P.E.: Proliferative endometrium; S.E.: Secretory endometrium; PCR: Polymerase chain reaction; IHC: Immunohistochemistry

Musashi-1, an RNA-binding protein in neural stem cells and epithelial progenitor cells that controls signalling pathways involved in self-renewal, is one such marker that has been studied within the endometrium. Musashi-1 expression was found to be present within epithelial cells of the endometrial glands and also within the stroma, and was seen to co-localise with its molecular target Notch-1 and telomerase. The cells positive for Musashi-1 were seen to be located more abundantly within the basalis when compared to the functionalis layer, and were significantly increased in number during the proliferative stage of the menstrual cycle over the secretory phase. High levels of Musashi-1 expression were also seen within tissue specimens taken from women with endometriosis and endometrial cancer (Gotte *et al*, 2008). A wide range of other commonly known stem cell markers including *OCT4, NANOG, KLF-4,BMI-1, SOX15, SALL4, UTF1* and in some of the literature *SOX2,* have all shown to be expressed within the endometrium (Bentz *et al*, 2010; Forte *et al*, 2009; Zhou *et al*, 2011; Gotte *et al*, 2011).

1.5.3 Specific Markers

OCT4, NANOG, SOX2

POU class 5 homeobox 1 (*OCT4),* Nanog homeobox (*NANOG)* and sex determining region Y-box 2 (*SOX2*) are all core regulators of human and mouse ESC pluripotency. They code for transcription factors for genes that control and preserve pluripotency within stem cells. Although broadly speaking all of these genes have a similar function, strictly speaking are each a little different. Levels of *OCT4* must be carefully balanced in order to maintain pluripotency, as low levels of expression are seen to induce trophectoderm differentiation, whereas over-expression triggers both endoderm and mesoderm differentiation. *NANOG* has been reported to repress embryonic ectoderm differentiation, but does not have any influence over other embryonic lineages. In contrast, *SOX2* is required for epiblast maintenance. Together these transcription factors form a network that regulates self renewal and pluripotency within stem cells (Wang *et al*, 2012; Forte *et al*, 2009).

The expression of *OCT4* has been identified within the endometrium of around 44% of women (Matthai *et al*, 2006). It has been shown not to vary with changes in the menstrual cycle and has been found mainly within the stromal cells of the endometrial and endometriotic samples with IHC (Bentz *et al*, 2010; Forte *et al*, 2009). *NANOG* has also been found within the endometrium and endometriotic tissue, although to a lesser extent than *OCT4*. It is also expressed in human endometrial adenocarcinoma (Forte *et al*, 2009; Zhou *et al*, 2011; Gotte *et al*, 2011). Gotte *et al* provided research which found *SOX2* expression within the endometrium at a 60-fold lower level than *OCT4* and *NANOG*. Gotte *et al* found that *SOX2*$^+$ stromal cells were significantly

raised within the proliferative phase, and found immunoflourescent co-localisation with telomerase, a stem cell marker associated with immortality (Gotte *et al*, 2011). In contrast, Forte *et al* did not find *SOX2* anywhere in the normal endometrium or endometriotic tissue (Forte *et al*, 2009).

CD133

CD133 is a 5-transmembrane glycoprotein that makes up a part of the prominin family of pentaspan membrane proteins. Its expression has been associated with HSCs within adult blood, the bone marrow and umbilical cord blood also. In addition, CD133 has been found on endothelial, neural and epithelial cells (Rutella *et al*, 2009). Recently, the expression of CD133 has been linked to malignancies of the prostate (Collins *et al*, 2005), lung (Eramo *et al*, 2008), brain (Singh *et al*, 2003) and ovaries (Ferrandina *et al*, 2008). Within childhood it has also been associated with acute lymphoblastic leukemia (Cox *et al*, 2009). CD133$^+$ cells taken from human colon cancer, have been shown to have the potential to give rise to tumours (Ricci-Vitiani *et al*, 2007). Following the emergence of evidence supporting the existence of cancer stem cells (CSCs) also referred to as tumour-initiating cells (TICs), CD133 is now widely considered as a marker for such cells, and has been used to isolate TICs in many solid tumours arising from different tissues (Rutella *et al*, 2009).

CD133 has been found within both the glandular and luminal epithelial cells of the normal endometrium using the monoclonal antibody AC141, which recognises the CD133 epitope (Schwab *et al*, 2008). CD133 expression has also identified within primary human endometrial tumours. CD133$^+$ cells possessed the ability to self-renew,

formed sphere-like structures in the correct culture environment and gave rise to tumours *in vivo* within immunocompromised mice. CD133$^+$ cells have an increased proliferative potential and tumourigenicity when compared to CD133$^-$ cells and showed apparent resistance to commonly used chemotherapy agents (Rutella *et al*, 2009; Nakamura *et al*, 2010).

PODXL

Podocalyxin (*PODXL*) is a type I transmembrane protein. It belongs to a large family of cell surface protein called sialomucins, and is closely related to the HSC marker CD34, and endoglycan. *PODXL* was first identified on normal kidney glomeruli, on the apical surfaces of glomerular epithelial cells called podocytes. Within the kidney, *PODXL* has been found to be important for kidney development, and is seen to control podocyte morphology and structural integrity (Nielsen *et al*, 2009; Doyonnas *et al*, 2005). Subsequently, it has been linked to haematopoietic progenitor cells, vascular endothelia and also a subset of neurones (Nielsen *et al*, 2009; Doyonnas *et al*, 2005). *PODXL* dysregulation has been implicated within a wide range of malignancies including breast (Somasiri *et al*, 2004), testicular (Schopperle *et al*, 2003), prostate (Casey *et al*, 2006), pancreatic (Ney *et al*, 2007) and hepatocellular carcinoma (Chen *et al*, 2004). *PODXL* has been identified as a marker for testicular malignancy within the human embryonic carcinoma cell line (Cheung *et al*, 2011) and has also found within all three germ layers during embryogenesis (Nielsen *et al*, 2009).

Although a recognised stem cell marker, *PODXL* has still not been linked to the endometrium within the literature. Recently within our lab, IHC has confirmed the

presence of *PODXL* within the vascular endothelium and the glandular secretions on the apical surfaces of glandular epithelial cells, within the normal endometrium. Staining intensity showed that *PODXL* expression was significantly higher within the proliferative phase compared to other phases of the menstrual cycle (unpublished data).

hTERT

Telomerase is a specialised ribonucleoprotein polymerase that catalyses the extension of telomeric DNA sequences at the chromosomal ends. By elongating the telomeres, telomerase activation provides cellular immortality and is thought to be an essential component of oncogenesis and the malignant transformation of most tissue types. The functional telomerase enzyme is composed of three major subunits; human telomerase RNA (hTR), telomerase protein 1 (TP1) and human telomerase reverse transcriptase (*hTERT*). It is the *hTERT* subunit which controls telomerase activity, and its mRNA expression is reported to correlate to telomerase activity levels as assessed using a telomere repeat amplification protocol (TRAP) assay (Paul-Samojedny *et al*, 2005; Kim *et al*, 2007). In most normal somatic cells, telomerase is usually inactive. However in cells which have a high regenerative capacity such as haematopoietic cells, cervical epithelial cells and normal endometrial cells, telomerase expression has been identified (Paul-Samojedny *et al*, 2005).

Within the endometrium, the level of telomerase activity has been found to be menstrual phase-dependent; highest within the proliferative phase and significantly suppressed within the secretory phase (Kyo *et al*, 1999). This telomerase activity has

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been localised specifically to the glandular epithelial component of the endometrium (Tanaka *et al*,1998). *hTERT* mRNA levels are also found to be significantly higher within endometrial cancer when compared to levels in the normal endometrium (Lehner *et al*, 2002). More recently, it was shown that *hTERT* expression is significantly increased within patients with endometriosis, and is further evidence to support that this benign condition resembles neoplastic disease (Kim *et al*, 2007). Due to its association with limitless replication potential, high telomerase activity is detected not only in cancer cells, but within stem cells also. In human keratinocytes, stem cells have been shown to be the main source of telomerase activity (Kyo *et al*, 1999).

LGR5

The leucine-rich repeat containing G protein-coupled receptor 5, *LGR5 (*also known as *Gpr49*), belongs to a family of receptors which are recognised by their seven-transmembrane-helical topology, including their extracellular N-terminus and intracellular C-terminus. These receptors have an important role in forming a connection between extracellular information and intracellular signal transduction pathways (Krusche *et al*, 2007). *LGR5* is now recognised as a marker for actively cycling stem cells in the small intestine, colon and hair follicle (Barker *et al,* 2007; Jaks *et al*, 2008). Intestinal epithelial *LGR5*^{+} cells, located at +4 position immediately above the Paneth cells in the crypt bases, have now been established as genuine intestinal stem cells. With a turnover time of 5 days, the intestinal epithelium is recognised as the fastest self-renewing tissue in mammals (Schepers *et al*, 2011). Independent *LGR5* knock-in mouse models also demonstrated the highly restricted

expression of this gene within a variety of adult tissues, such as the gastric and mammary glands, suggesting that it may also be a more general marker of ASCs (Barker *et al*, 2007; Barker *et al*, 2010). The human endometrium resembles the gastrointestinal system with its rapid turnover time, highly regenerative capacity and glandular components. Its *LGR5* mRNA has been found in the endometrium throughout the menstrual cycle and within cultured endometrial epithelial cells (Krusche *et al*, 2007).

ERα, ERβ and *PR*

The ovarian hormones, oestrogen and progesterone, are key ingredients for the physiological processes that occur within the endometrium. These work by binding to and activating the receptors, *ER* and *PR*. For many years it was believed that only single receptors for *ER* and *PR* existed. However, recently evidence has emerged for two major isoforms for both *ER* (α and β) and *PR* (A and B) exist (Mylonas *et al*, 2007).

Oestrogen controls many fundamental processes that occur within the endometrium including proliferation and vascularisation, and also up-regulates various genes including *PR*, vascular endothelial growth factor (VEGF) and lactoferrin. *ERα* and *ERβ* are highly homologous, apart from the C-terminal ligand binding domain and N-terminal transactivation domain (AF-1) which differ. Both isoforms bind to E2 with high affinity. *ERα* expression rises within the glandular and stromal cells of the proliferative functionalis layer, and drops during the secretory phase. The expression of *ERα* is not seen to fluctuate throughout the menstrual cycle within the basalis.

Although the role of *ERβ* within the endometrium is still unclear, unlike *ERα,* it is detected in and therefore associated with the endometrial vascular endothelial cells (Critchley *et al*, 2006; Brosens *et al*, 2004; Critchley *et al*, 2009).

PR expression is induced during the proliferative phase of the cycle, driven by oestrogen. *PR-A* is the shorter of the two isoforms and is homologous with *PR-B* except for that it is missing the last 164 amino acids that are present at the end of the B subtype. The expression of both *PR* isoforms is significantly higher within the endometrial glands of the functional layer during the proliferative phase of the cycle, compared with the secretory phase. However, *PR* expression is seen to persist within the stroma of the functional layer, especially in the cells located closely to the uterine vasculature, with *PR-A* acting as the dominant receptor within these cells. The cells within the basalis appear to be controlled differently as *PR* expression does not fluctuate within the glands and stroma of this layer. *PR* expression also varies between the epithelial and stromal cell types. For example within the secretory phase, both isoforms of *PR* are reduced within the epithelial cells of the functional layer, however only *PR-B* declines within the stroma of the same layer (Critchley *et al*, 2006; Critchley *et al*, 2009).

Regeneration of the endometrium is controlled by the ovarian hormones mentioned above and is said to stem from SPCs within the basal layer. Prianishnikov in 1978, suggested a theory that still stands today. He claimed that the proliferation of endometrial stem cells occurs independently of hormones, which lack the ovarian hormone receptors. Daughter cells that arise from these become hormone-sensitive in

the presence of oestrogens and acquire *ERs* enabling them to respond to oestrogen. With further replication, these cells later acquire *PRs* and become sensitive to both hormones. With time, as the cells mature further and move into the secretory phase of the menstrual cycle, they become unresponsive to oestrogen but retain their *PR*s, become more differentiated and exhibit secretory changes. According to this proposed model, *ER* and *PR* are therefore both markers of more differentiated endometrial cells, with *PR* acting as a marker of greater differentiation than *ER* (Prianishnikov, 1978).

CD9

Another marker that is important within the endometrium is CD9. CD9 is a 24-27 kD glycoprotein that was discovered by Park *et al* to be strongly expressed within the glandular and luminal epithelial cell population of the endometrium equally throughout all phases of the menstrual cycle. Stromal cells were seen negative for CD9. CD9 is closely linked to integrins and is important for cell adhesion and motility, and therefore may have a role in blastocyst implantation and trophoblast invasion. Since this discovery was made, CD9 has been adopted by many endometrial studies as a main stream surface marker for the identification of endometrial epithelial cells. CD13, also known as aminopeptidase N, has also been established as the equivalent surface marker for the endometrial stromal population (Park *et al*, 2000; Kato *et al*, 2007).

1.6 Stage Specific Embryonic Antigen-1 (SSEA-1) and the Endometrium

As previously described, much work has been dedicated to the identification of markers which characterise the stromal stem cell. As yet however, no such markers

have been identified for endometrial epithelial SPC population. Our lab has invested time and resources into the research and identification of such markers. In recent unpublished data, our lab has identified a significant and exciting link between these cells and the surface marker, stage specific embryonic antigen-1 (SSEA-1) (Valentijn *et al*, 2013).

SSEA-1, also referred to as Lewis-X (LeX) and CD15, is a carbohydrate moiety located on the cell surface. It is expressed by the pluripotent mouse blastocyst, ESCs and primordial germ cells (Capela *et al*, 2006). Within mice, SSEA-1 is present in embryonic cancer cells (ECCs) and within late 8-cell embryos. In mice ESCs its expression is weak within the early stages and becomes more prominent later on. In contrast, SSEA-1 is not expressed within human ESCs or ECCs, but instead they express the carbohydrate antigens SSEA-3 and SSEA-4. Hence within humans, SSEA-1 is described as an ESC early differentiation marker (Muramatsu *et al*, 2004; Scaffidi *et al*, 2011). In addition, SSEA-1 expression has been linked to progenitor and stem cells in a range of different adult tissues, including the central nervous system, cardiovascular system and ciliary epithelium of the eye (Capela *et al*, 2006; Blin *et al*, 2010; Koso *et al*, 2006). SSEA-1 is also recognised as a marker for tumour-initiating cells within studies and has been identified as a marker for cancer stem cells within the brain (Son *et al*, 2009; Scaffidi *et al*, 2011). Within these tissues the function of SSEA-1 has been linked to processes including blastomere adhesion, cell-cell interaction and growth factor binding (Muramatsu *et al*, 2004; Capela *et al*, 2006). As SSEA-1 is a fucose-containing trisaccharide, it is must be glycosylated by a group of enzymes called fucosyltransferases (FucTs). The role of FucT enzymes is to transfer fucose in

α1, 2, α1,3/4 and α1,6 linkages on to a number of glycans and is crucial for the production of Lewis systems. Fucosyltransferase 4 (*FUT4)* is one of these enzymes that regulates carbohydrate antigen expression and whose function has been linked to blastocyst attachment. Also linked to leukocyte adhesion, *FUT4* was the first leukocyte-associated enzyme that was associated with the synthesis of ligands including LeY and LeX, or SSEA-1. Investigations into *FUT4* within the endometrium have shown distinct cyclical changes and hormonal regulation of *FUT4* mRNA to support that it is up-regulated during the period of implantation and is regulated by progesterone. These findings strongly suggest that *FUT4* is responsible for the final catalysing step in the production of SSEA-1 (Ponnampalam *et al*, 2008). Within the nomenclature, *FUT4* is even provided as an alternative name for SSEA-1.

Carbohydrate antigens on the surface of stem cells are useful to enable the isolation and identification of such cells, and act as excellent biomarkers. To date, SSEA-1 has never been linked to the endometrium and its exact function within the endometrium is still unknown. Within our lab, research has shown that SSEA-1$^+$ epithelial cells within the endometrium, exhibit many characteristics of SPCs. IHC of full thickness endometrium taken from healthy women, shows positive staining for SSEA-1 exclusively within the glandular and luminal epithelium, but not within the stromal compartment. Staining shows the presence of SSEA-1 strongest within the basal glands of pre-menopausal women, where endometrial SPCs are thought to arise, and has proven to be menstrual-phase dependent with more intense basal staining correlating with the early proliferative phase of the cycle. Strong staining is also evident within the endometrium of post-menopausal women, in whom only the basalis

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layer of the endometrium remains. Not only is SSEA-1 present within the correct location for epithelial SPCs within the normal endometrium, but SSEA-1 also stains positively within eutopic and ectopic endometriotic tissue and within endometrial cancer cells, the pathogeneses of which are related to abnormal stem cells. See figures 1.7 and 1.8 below (Valentijn *et al*, 2013).
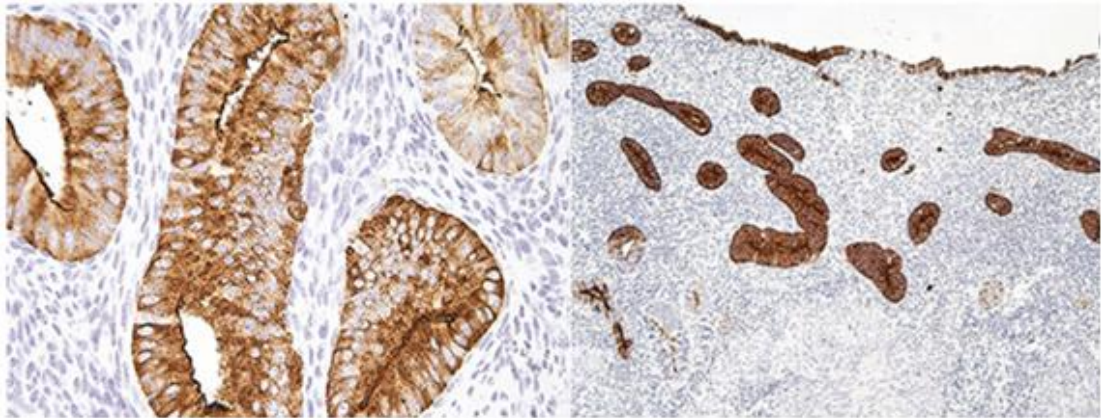
Figure 1.7 Positive immunostaining for SSEA-1 within the basal glands of a normal cycling woman taken in the proliferative phase (left) and within the basal layer of the postmenopausal endometrium (right) (Valentijn *et al*, 2013)
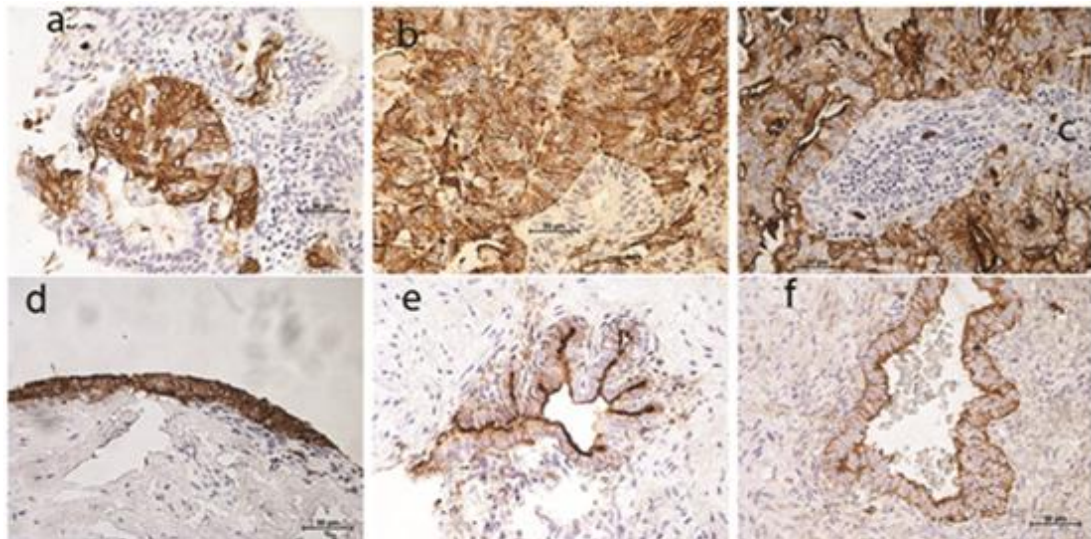
Figure 1.8 Positive immunostaining seen within endometrial cancer grades 1(a), 2(b) and 3(c) and superficial (d) and deep (e & f) ectopic endometriotic lesions (Valentijn *et al*, 2013)

Endometrial epithelial SSEA-1$^+$ cells also exhibit high proliferation potential as shown by significant expression of the proliferation marker Ki67 and the mitotic marker phosphohistone H3. Investigation using the TRAP assay revealed that SSEA-1$^+$ epithelial cells demonstrate significantly higher telomerase activity and telomere lengths over their SSEA-1$^-$ epithelial counterparts, both features of activated stem cells. See figure 1.9 below (Valentijn *et al*, 2013).
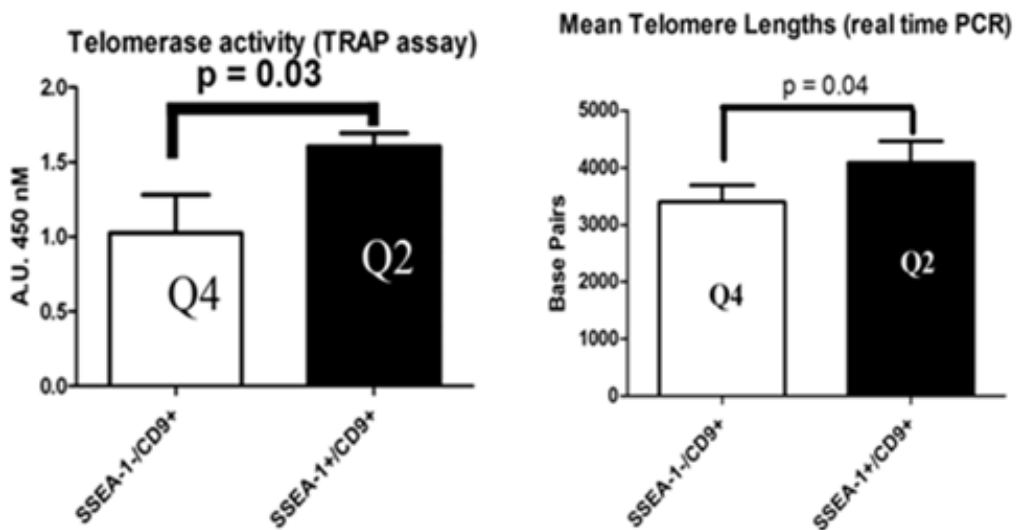
Figure 1.9 SSEA-1$^+$/CD9$^+$ endometrial cells exhibit significantly higher telomerase activity and mean telomere lengths than SSEA-1$^-$/CD9$^+$ endometrial cells (Valentijn *et al*, 2013)

Within 3D culture, which recapitulates the stem cell niche and favours stemness, human endometrial epithelial cells have been observed to form hollow gland-like structures like those seen within the normal endometrium. Stromal cells are not seen to form these spheroid structures in culture. SSEA-1$^+$ endometrial epithelial cells show a significantly increased tendency to produce a higher number of spheroids than the SSEA-1$^-$ epithelial cells. They retain SSEA-1$^+$ expression until they mature and differentiate when expression is restricted to only a few cells. See figure 2.0 below (Valentijn *et al*, 2013).

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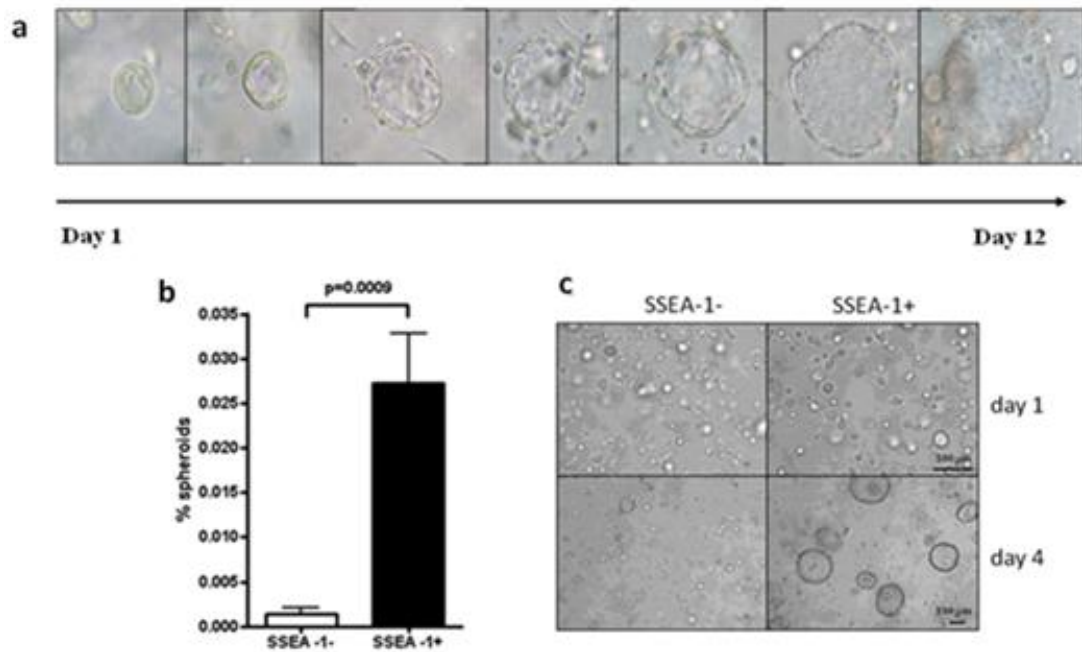
Figure 2.0 a) Micrographs to show spheroid growth and formation over a 12 day culture period in Matrigel, images shown for b) & c) SSEA-1⁺ epithelial population give rise to a significantly higher proportion of spheroids by day 7 (b) and day 4 (c) respectively (Valentijn *et al*, 2013)

This evidence suggests that that SSEA-1 may be a potential marker for enriching endometrial epithelial SPCs in humans and that a subpopulation of SSEA-1$^+$ may contain epithelial SPCs. As previously discussed, in order to establish a SPC population, other functional properties including self-renewal, differentiation and *in vivo* behaviour must be evaluated on the SSEA-1$^+$ epithelial cell population. In addition, to further characterise these cells, their gene expression profile must be evaluated to assess whether they possess markers of stemness which would suggest stem cell-like behaviour. Gene expression analysis on these cells is therefore the focus of this study, and may provide further evidence to suggest that SSEA-1 marks an epithelial SPC population within the endometrium.

1.7 Clinical Implications and Endometriosis

The identification, isolation and characterisation of endometrial SPCs have many important clinical implications. Stem cell therapy, as already mentioned, is seen as one of the most promising techniques for the future of medicine and regenerative therapy. Cell therapy can be used to target a wide range of diseases which may affect any part of the human body. The endometrium may act as an easily accessible and renewable source of such cells. In addition, abnormal endometrial stem cells are postulated to be involved in the pathogenesis of gynaecological diseases such as endometriosis, endometrial hyperplasia, endometrial cancer and adenomyosis, in association with abnormal endometrial proliferation. Therefore the identification of endometrial SPCs may not only be used to treat other medical conditions, but will increase our understanding of the aetiology of gynaecological conditions such as endometriosis and hence their treatment also (Aplin, 2008).

1.7.1 Endometriosis

Endometriosis is a benign chronic gynaecological condition, defined by the existence of endometrial glands and stroma outside of the uterine cavity. It is one of the most common conditions seen within gynaecology. It is a common disorder affecting 6-10% of all reproductive-aged women, and 35-50% of women who present with the classical symptoms of pelvic pain and infertility. There are no differences in the incidence of this disease across different races, although Japanese women have been observed to have twice the incidence of Caucasian women. The clinical presentations of endometriosis may vary from patient to patient however, with some experiencing severe symptoms such as dysmenorrhea and dyspareunia, while others remain

asymptomatic. Ectopic endometrial tissue is most commonly found on the pelvic peritoneum and ovaries, as well as within other pelvic structures such as the fallopian tubes, bladder, colon, rectovaginal septum and sacrouterine ligaments. Implants have additionally been located at more distant sites such as the pericardium, pleura, lung parenchyma and even the brain. Although not life threatening, endometriosis has a major economic and social impact. Population-based studies have also discovered that women suffering from endometriosis are at an increased risk of developing ovarian cancer (Sasson *et al*, 2008; Overto *et al*, 2007).

Pathogenesis of Endometriosis

Despite its common occurrence, the pathogenesis of endometriosis remains unknown and has been under extensive investigation for a long time. A number of hypotheses exist regarding its aetiology, the most common and widely accepted of these being Sampson's theory of retrograde menstruation. Sampson postulated that ectopic implants arise due to menstrual debris migrating through the fallopian tubes and into the peritoneal cavity where they adhere to and invade the peritoneal mesothelium, establishing endometriotic lesions. On the other hand, it is thought that 90% of menstruating women contain this debris within their peritoneal cavities and therefore it is likely that only some of these cells are capable of giving rise to endometriosis in certain women. An altered peritoneal environment along with abnormalities in genetic, immunologic and environmental factors may also have an influence on their survival and proliferative capacity. Strong links between endometriosis and multiple gene loci, immune deficiency and also various environmental factors have now been established (Figueira *et al*, 2011; Sasson *et al*, 2008; Aplin *et al*, 2008).

Other theories include the embryonic rest theory, the lymphovasular metastasis theory and the coelomic metaplasia theory. The embryonic rest theory suggests that endometriosis is developed from scattered embryonic rests of műllerian origin, which when subjected to the correct stimuli, can form endometrial tissue. Alternatively, the lymphovascular metastasis theory claims that endometriosis occurs due to the lymphovascular spread of menstrual tissue, accounting for the occurrence of endometriosis in distant sites such as the lungs and brain. Finally, the colomic metaplasia theory suggests that it is the spontaneous metaplasia of the pleural and peritoneal mesothelial cells that leads to endometriosis. Although evidence exists for each of these theories, it is probably linked to a combination of abnormal biological process (Figueira *et al*, 2011; Sasson *et al*, 2008).

Diagnosis of Endometriosis

Being an oestrogen dependent disorder, endometriosis usually affects women during their reproductive years when the lesions are stimulated by the ovarian hormones. Symptoms are usually the strongest pre-menstrually and are relieved following menstruation. Patients may complain with a variety of symptoms, the most common of which being pelvic pain. Other symptoms include back pain, dyspareunia, dyschezia and pain with micturition. For some, the only presenting complaint may be a history of infertility. A family history of endometriosis in a first degree relative may also give some indication as to the diagnosis. A thorough patient history can indicate endometriosis as a differential but not a definitive diagnosis. It is often misdiagnosed as irritable bowel syndrome. On physical examination, signs may be absent, or might

present as tender nodules in the posterior fornix or adenexa, cervical motion tenderness or even a fixed retroverted uterus (Mounsey *et al*, 2006).

The gold standard method of making a diagnosis of endometriosis is by direct visualisation of the ectopic lesions, usually via laparoscopy. This is commonly followed by histological confirmation, in which at least two features are present. These features include hemosiderin-laden macrophages, endometrial epithelium or stroma. Histologically, these lesions are similar to the eutopic endometrium. Transvaginal ultrasonography is used as a valuable investigation to locate retroperitoneal and uterosacral lesions, however unfortunately it does not accurately identify peritoneal lesions or small endometriomas. Two other tests, including serum cancer antigen 125 (CA 125) and magnetic resonance imaging (MRI), have both been studied for their use in endometriosis, however neither has shown diagnostic accuracy. It is important that non-gynaecological causes of pelvic pain are also excluded (Mounsey *et al*, 2006; Bergqvist *et al*, 1984).

When diagnosing a patient with endometriosis, a clinical staging system is required in order to allow clinicians to communicate effectively with one another regarding the severity and management. Such a system was revised by the American Society for Reproductive Medicine (ASRM) in 1996 and is the most widely accepted system, although it does not act as a sensitive predictor for pregnancy following treatment and does not link the level of pain with the staging severity (see table 2) (The Practice Committee of the ASRM, 2006; ASRM, 1997).

Table 2 Revised ASRM classification for endometriosis, 1996 (ASRM, 1997)

Stage	Description
I	Minimal: Superficial lesions only and may have few adhesions
II	Mild: Stage I and some deep lesions within the cul-de-sac
III	Moderate: Stage II, identification of ovarian endometriomas and increased adhesions
IV	Severe: Stage III, larger endometriomas and extensive adhesions

Treatment of Endometriosis

The treatments for endometriosis encompass both medical and surgical techniques, and focus on relieving pain, preventing disease progression and promoting fertility. The choice of management must be made according to a patient's individual needs, taking into consideration the severity of their symptoms, their age and their desire for a family. The management available for endometriosis is not curable. In many women, the menopause can naturally treat the disease. Several pharmacological options are available and are aimed at managing symptoms of pain, and cyclical dysfunction. Non-steroidal ant-inflammatory drugs (NSAIDs) provide pain relief, whilst the oral contraceptive pill (OCP), androgenic agents (e.g. danazol), progestogens and GnRH analogues all manage pain whilst simultaneously inducing amenorrheoa. Medical management is limited with its use due to their long term side effects and reccurrence of endometriosis is common following their discontinuation. Surgical interventions provide an alternative, including laparoscopic resection/ablation, which have shown to help increase a woman's fertility. However the effects of surgery may be short lived, as within 12 months symptoms of pain reoccur in almost 50% of women and further medical treatment is required. More definitively a hysterectomy and bilateral salpingoophorectomy can remove symptoms, although this may not be an option for

women who still want a family (Mounsey *et al*, 2006; Overto *et al*, 2007; The Practice Committee of the ASRM, 2006).

1.8 Research Aims and Objectives

1.8.1 Research Aims

There are still many unanswered questions about the aetiology surrounding endometriosis, and the treatments available are far from ideal. One hypothesis regarding the pathogenesis of endometriosis claims that endometrial SPCs are abnormally shed into a woman's peritoneal cavity during menstruation, where they adhere and proliferate forming ectopic lesions. An alternative thought is that endometrial SPCs may be intrinsically abnormal in women with endometriosis, giving them a greater ability to implant and form lesions (Gargett *et* al, 2010). As yet, unlike the stromal population, no markers have been established for endometrial epithelial SPCs which would allow their identification and location within the tissue, and would enable their isolation and further study. Prospective isolation of endometrial SPCs may provide exciting possibilities for the future of medicine, allowing for their use in regenerative medicine throughout the body and an increase in our understanding of gynaecological disorders hence improving their treatment.

In our lab, SSEA-1 has been identified as one potential marker located on endometrial epithelial SPCs, as these cells seem to possess many qualities that SPCs should have. Further evidence is required into the study of the SSEA-1$^+$ endometrial epithelial cell population in order to definitively conclude that it is an epithelial SPC marker. One important area which will allow the further characterisation of these cells and has yet

to be defined, is their transcriptional profile for common endometrial markers and markers of stemness which control stem cell properties such as self-renewal and the maintenance of an undifferentiated state. This may or may not provide further evidence for their SPC-like qualities, and therefore is the main focus of this study.

1.8.2 Objectives

In order to achieve this broad research aim, several specific objectives have been identified below to be achieved throughout the course of the study.

Assess the gene expression profile for SSEA-1$^+$ epithelial cells versus SSEA-1$^-$ epithelial cells within the normal endometrium, cultured in a normal 2D system. In order to do this, quantitative PCR (qPCR) was performed on both cell populations to compare mRNA expression of the commonly recognised stem cell markers *OCT4, NANOG, SOX2, PODXL, hTERT, CD133* and *LGR5*, and of the endometrial differentiation markers *ERα, ERβ* and *PR*. Additional markers *CD9*, as an epithelial cell marker, and *FUT4*, as a marker for SSEA-1, have also been included within this study for extra value.

Assess the gene expression profile for SSEA-1$^+$ epithelial cells versus SSEA-1$^-$ epithelial cells within the normal endometrium, cultured in a 3D Matrigel system.
SSEA-1 sorted endometrial epithelial cells form gland-like structures in 3D Matrigel, similar to the glands seen within the normal endometrium. This 3D culture system is said to mimic the endometrial stem cell niche and therefore may favour stemness and

prevent differentiation. It is therefore important to assess the gene expression profile, looking at the same genes, on SSEA-1$^+$ and SSEA-1$^-$ cells grown in 3D culture to assess if there are any differences with 2D culture.

Compare the gene expression profile for SSEA-1$^+$ epithelial cells taken from women with a normal endometrium to those with endometriosis.

As endometriosis is said to have a link with abnormal endometrial SPCs, it is important to evaluate whether cells, which express our proposed SPC marker SSEA-1, show any differences in their gene expression within patients suffering from endometriosis. The same genes as previously mentioned was evaluated in both the 2D and 3D culture systems on these patients

Chapter 2

Materials and Methods

Chapter 2: Materials and Methods

All equipment and reagents have been highlighted as 'bold' within the text. The specifications including the company name and catalogue number of each item can be found listed in alphabetical order in appendix II.

2.1 Ethics Approval

All patients included in the study had given informed written consent prior to collection. Ethical approval for the study was granted by the Liverpool (Adult) Research Ethics Committee (LREC) (reference 09/H1005/55 and 04/Q1505/112). This approval allowed the collection of human endometrial tissue samples from all suitable patients, including healthy fertile women and women with endometriosis, who were attending the Liverpool Women's Hospital (see appendix III).

2.2 Patient Recruitment and Sample Collection

2.2.1 Patient Identification and Recruitment

Suitable patients undergoing gynaecological surgery were identified following review of the planned theatre lists. Once these patients were highlighted, their paper-based hospital notes were cross-referenced in order to ensure that they met the correct inclusion criteria (see table 3). Suitable patients were provided with a detailed description of the purpose of the study and the procedure itself, including any potential risks or benefits to the patient. To guarantee informed valid consent, this information was provided by staff who were trained in Good Clinical Practice (GCP) and were able to consent and recruit the patients for this study. All patients were made aware that they had the right to refuse participation within the study and were ensured that this

would not affect their treatment. In addition, patients were also provided with an information leaflet to read in their own time (appendix IV) and were given the opportunity to ask any questions they may have. Once verbal consent was given, staff could then proceed to gain written consent (appendix V). The following demographic details were collected for each patient: participant age, weight (kg), height (cm), body mass index (BMI), smoking history, parity, history of miscarriage or termination of pregnancy, the date of last menstrual period (LMP), cycle length and the number of days of menstruation, and their past medical history (see appendix VI).

2.2.2 Inclusion/Exclusion Criteria

The study included two groups of patients. The inclusion and exclusion criteria specified below were employed in order to select and recruit patients into the study (tables 3 and 4).

Table 3 Control Group Inclusion/Exclusion criteria

Control Group: Fertile women within reproductive age **(n=14)**

Inclusion Criteria	*Exclusion Criteria*
• Women of reproductive age • Women undergoing surgery for benign gynaecological conditions (e.g. laparoscopic sterilisation or uterine fibroids)	• Postmenopausal women • Women on hormonal therapy within the last 3 months • Pregnant or breastfeeding women • History of infertility • History of endometriosis or gynaecological malignancy

Table 4 Endometriosis Group Inclusion/Exclusion criteria

Endometriosis Group: Women with active endometriosis **(n=8)**

Inclusion Criteria	*Exclusion Criteria*
• Women of reproductive age • Women with a surgical diagnosis of active eutopic or ectopic endometriosis at the time of sample collection	• Women on hormonal therapy within the last 3 months • Pregnant or breastfeeding women

2.2.3 Endometrial Biopsy Collection

Endometrial biopsies were collected by trained professionals who have been trained in the correct method of sample collection from theatre. Depending on the type of the surgical procedure planned for the individual, we employed one of two following methods to attain an endometrial biopsy.

1. **Full Thickness Sampling:**

   Full thickness endometrial samples were collected from women undergoing a hysterectomy. Following surgical removal of the uterus, a single vertical midline incision was made on the posterior surface of the uterus from the fundus down to the cervical
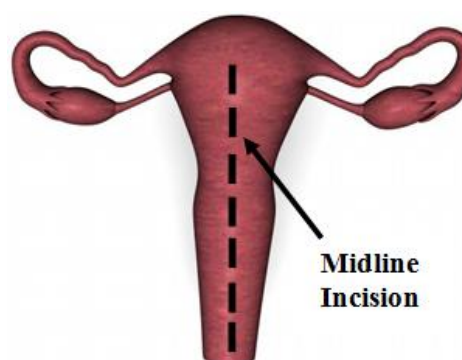
Figure 2.1 Full Thickness Endometrial Sampling

   canal (excluding the cervix) using a **size 22 carbon steel surgical blade** (indicated in figure 2.1). This exposed the inner uterine cavity and the endometrium. A lateral incision (approximately 25mm wide and 10mm deep)

was then made into the uterine wall allowing a full thickness sample to be obtained which comprised of the endometrial lining, the endometrial-myometrial junction and part of the myometrium.

2. Pipelle Sampling:

For women who were not having a hysterectomy, we collected a pipelle endometrial biopsy under anaesthetic before their planned operation had begun. This is a common and relatively non-invasive method of endometrial sampling, which is commonly used in the outpatient clinic setting. A pipelle is a flexible, blunt plastic tube which collects endometrial tissue through suction. Once on the operating table, patients were placed in the lithotomy position and a sterile aqueous solution containing **chlorhexidine gluconate 0.05%** was used in order to clean the perineal area externally and the vagina and the cervix internally as a part of routine gynaecological surgical preparation. Following this, a Sims speculum was inserted into the vagina in order to visualise the cervix (this was routinely required as part of the gynaecological surgical procedure regardless of whether the patient was in our study, therefore was not over and above what was clinically needed for her). Then, a pair of **Teales Vulsellum uterine forceps** was used to hold the anterior lip of the cervix and a pipelle was inserted in through the external and internal cervical os towards the fundus of the uterine cavity. Superficial endometrial tissue was collected by the use of rotation and backwards and forwards movements of the pipelle, and creating suction by drawing the plunger. Once the necessary amount of endometrial tissue was withdrawn, the pipelle was removed completely from the uterine cavity.

These tissue collection methods ensured safe, sterile conditions and caused minimal discomfort to the patient and therefore in our opinion was ethically acceptable.

2.2.4 Sample Processing

Once fresh tissue samples had been collected, they were divided into smaller fragments for transfer into multiple storage containers in preparation for subsequent laboratory experiments. For our study it was essential to collect primary human endometrial tissue (without the underlying myometrium) within collection media. For cell culturing, biopsies were collected in to **Dulbecco's Modified Eagle Medium/F12 (DMEM/F12)**, 1% **Fetal Bovine Serum (FBS)** and 0.2% **Primocin** (collection medium). This was kept at 4°C until ready for subsequent cell culture.

2.3 Isolation of SSEA-1 Enriched and Depleted Populations

2.3.1 Endometrial Epithelial Cell Isolation

Biopsy tissue that had been placed in collection media was transferred to a **100mm Petri dish** along with 1ml of DMEM/F12 to maintain tissue moisture. A surgical blade was used to chop up the endometrial tissue into a fine mince consistency. Following this, the tissue was transferred into a **30ml universal tube** using a **1ml pastette** and the Petri dish was rinsed with DMEM/F12 to ensure that all remaining tissue was collected. The tube was centrifuged at 500g for 5 minutes to collect the cells in pellet form. The cell pellet was then resuspended in 4ml DMEM/F12, 500µl 1x **collagenase (20mg/ml)**, 100µl 1x **dispase (10mg/ml)**, 100µl 1x **DNase (4mg/ml)** and 50µl 100mM **MgCl$_2$**.The suspension was incubated for 90 minutes in a shaking water bath at 37°C to allow digestion into single cells. During this time period, the digest was triturated periodically to dissociate digested tissue.   Following this the digest was filtered

through a **40μm cell strainer** into a **50ml universal tube**, and the filter was washed thoroughly with around 30-40ml DMEM/F12. The epithelial glands which were too big to pass through were retained at the base of the filer, and were named the 'retentate'. The stromal cells and contaminating red blood cells which were passed through the filter were named the 'flow through'. The retentate was then back-washed using 30-40ml of DMEM/F12 into a 50ml tube, by inverting the cell strainer. Both fractions were then centrifuged at $500g$ for 5 minutes to pellet cells.

For the stromal cell fraction:

The stromal cell pellet was then resuspended in 4ml DMEM/F12 and layered on to 4-5ml of **Ficoll** in a **15ml centrifuge tube**. This was subsequently centrifuged at $400g$ for 10 minutes to pellet the contaminating red blood cells. Following this, the stromal cells appeared as a single layer at the interface between the Ficoll and the media and these were transferred carefully into a 30ml universal tube. The stromal cells were then washed with 15ml 1x DMEM/F12 and centrifuged at $500g$ for 5 minutes. The stromal cells were then left on ice.

For the epithelial cell fraction:

The epithelial cell pellet was resuspended in 1ml DMEM/F12, 1ml 0.25% **trypsin/EDTA solution** (0.125% final concentration), 100μl 1x DNase (4mg/ml) and 50μl 100mM $MgCl_2$ in a 15ml centrifuge tube. This was incubated at $37^{o}C$ for 20 minutes and triturated in order to break apart the epithelial glands and liberate the single epithelial cells. 1ml of culture medium was then added to inactivate the trypsin. The epithelial cell fraction was centrifuged at $500g$ for 5 minutes and then washed in x1 DMEM/F12 in a 30ml universal tube.

For both stromal and epithelial cell fractions, the cell pellets were resuspended in 10ml of culture (or complete) medium (defined as DMEM/F12, 10% FBS and 50ng/ml **epithelial growth factor(EGF)**) and were plated in either **tissue cultureT75cm^2 flasks** or 100mm Petri dishes, depending on the cell yield obtained. This was incubated for 20-30 minutes at 37°C. Selective adherence was used to further enrich the epithelial and stromal fractions. Unlike epithelial cells which take longer to attach, stromal cells attach quickly and readily and therefore adhesion can be monitored with the use of an inverted microscope. Non-adherent epithelial cells were transferred to a new culture vessel. Selective adherence was repeated if necessary to achieve optimal enrichment. Cells were cultivated for up to 3 days, depending on the confluency. The morphology of the final cell fractions are shown below in figure 2.2.
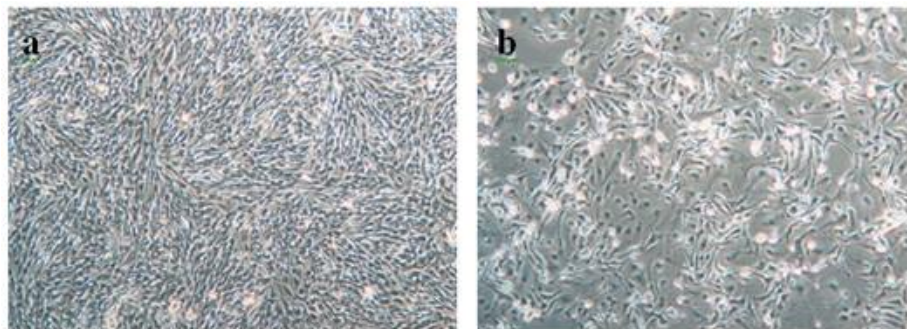
Figure 2.2 Endometrial stromal and epithelial cell morphology **a) stromal cells:** characteristic tight spindle shape morphology; **b) epithelial cells:** typical polyhedral/whorled morphology (provided by Anthony Valentijn, Liverpool Women's Hospital)

The purity of the epithelial cell fraction could be assessed morphologically as described above, but also with the use of FC and immunofluorescence (IF) for the epithelial marker CD9. The following FC data demonstrates that on days 3-5 of culture, greater than 80% of the cells were epithelial in origin (CD9$^+$) with less than 20% stromal cell contamination (CD13$^+$) (figure 2.3).
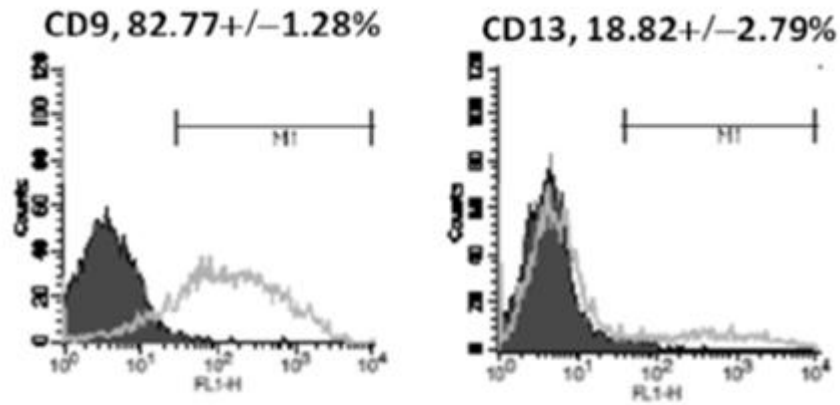
Figure 2.3 FC analysis of endometrial epithelial cells. CD9 epithelial cell marker; CD13 stromal cell marker (provided by Anthony Valentijn, Liverpool Women's Hospital)

FC analysis also confirmed that other cell types including endothelial cells (CD31^{+}), haematopoietic stem cells (CD34^{+}) and leukocytes (CD45^{+}) were also present in extremely low numbers (less than 3%) (figure 2.4).
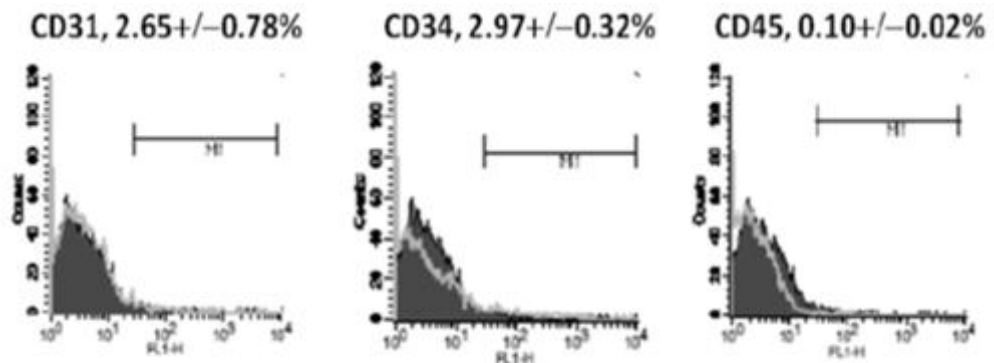
Figure 2.4 FC analysis of endometrial epithelial cells. CD31 endothelial cell marker; CD34 haematopoietic stem cell marker; CD45 leukocyte marker (provided by Anthony Valentijn, Liverpool Women's Hospital)

2.3.2 Cell Sorting

Magnetic-Activated Cell Sorting (MACS)

Following epithelial cell enrichment, **anti-SSEA-1(CD15) MicroBeads** were used to sort the epithelial cell population on the cell surface marker, SSEA-1. The MACS sorting of these epithelial cells into SSEA-1^{+} depleted and enriched cell fractions was

performed according to manufacturer's instructions. Cell culture medium was first aspirated and the 100mm Petri dish was washed with **phosphate buffered saline (PBS)**. Subsequently the cells were trypsinised by using 1ml of 0.05% trypsin/ EDTA solution and incubated at 37°C for 5 minutes. Following this, 2ml of culture/complete medium (containing FBS) was added to stop the reaction and inactivate the trypsin. To break apart any cellular aggregates and form a single-cell suspension, the solution was triturated before transfer through a 40μm cell strainer which had been placed over a **50ml Falcon tube**. To recover the cell pellet, the cell suspension was then centrifuged at 500g for 5 minutes at 4°C. Ideally up to 10^{7} cells were recovered at this point. The cell pellet was then resuspended in 1ml MACS buffer (composed of PBS, 0.5% **bovine serum albumin (BSA)** and 1mmol **ethylenediaminetetraacetic acid (EDTA)** and was transferred to a pre-chilled **1.5ml microcentrifuge tube**. This step washed the cells and exposed them to the same conditions as used later for antibody binding, before further centrifugation at 700g for 3 minutes. The supernatant was removed and the cell pellet gently resuspended in 80μl of buffer and 20μl of anti-SSEA-1(CD15) MicroBeads. This was mixed well and incubated at 4°C for 20 minutes in a refrigerator to allow magnetic labelling of the cells. The microcentrifuge tube was flicked periodically to ensure that any settled cells were resuspended. This cell suspension was washed in 1ml of Miltenyi buffer and centrifuged at 700g for 3 minutes as before. This step was then repeated for a second time following aspiration of the supernatant. In order to magnetically separate the labelled cells, a **MACS separation column** was placed in a magnetic field using a MACS separator. The separation column was prepared by adding a **30μl yellow pre-filter** and pre-wetting it with 500μl of buffer. 500μl of this buffer was also added to the cell pellet following aspiration of the supernatant, before loading the cell suspension onto the column. The cell suspension

was washed through the column with 500µl of buffer twice, and this 'flow through' was collected into a 1.5ml microcentrifuge tube containing the unlabelled SSEA-1$^+$ depleted cells. The column was subsequently washed for a third time with 500µl of buffer, however this 'waste' solution was discarded into a separate tube. The column was then removed from the magnetic cell separator and placed on a third tube before washing it with 1.5ml of buffer to collect the 'eluate' containing the magnetically labelled SSEA-1$^+$ enriched cells. Care was taken to only remove the magnetic field once the column was carefully placed over the collection tube. Finally, in order to recover the cell pellets from the 'eluate' and 'flow through' cell suspensions, both fractions were centrifuged at 700g for 8-10 minutes to collect as many cells as possible.

Magnetic labelling of SSEA-1$^+$ cells   Magnetic separation using a MACS column placed in a MACS separator   Magnetically labelled SSEA-1$^+$ cells flushed out once the MACS column removed from magnetic field
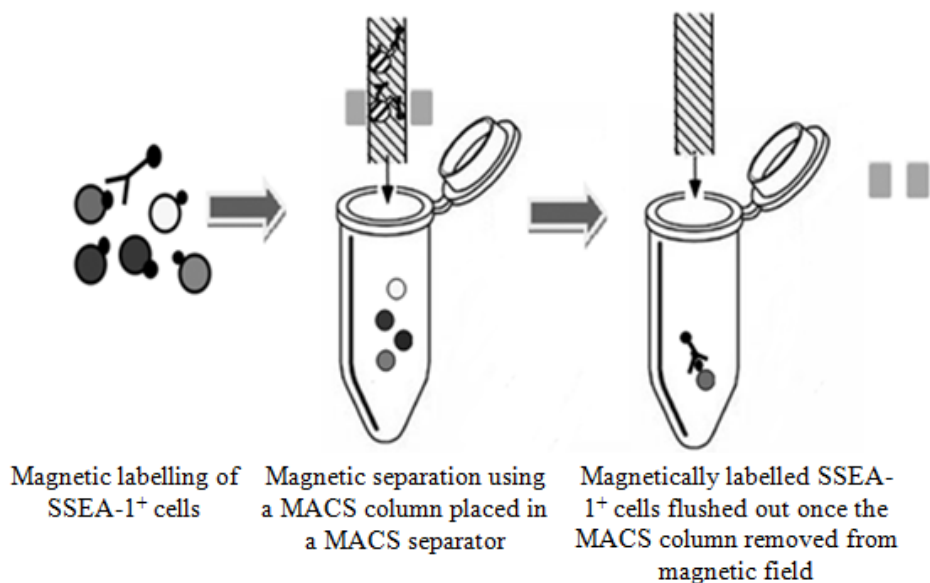
Figure 2.5 Schematic of MACS sorting

Western blotting confirmed that the SSEA-1$^+$ fraction was indeed enriched for SSEA-1 (figure 2.6). With the use of FC analysis, expression of SSEA-1 was routinely seen within 20-30% of the cultured epithelial cells. Stromal cells did not appear to express SSEA-1, as evidence by IHC.
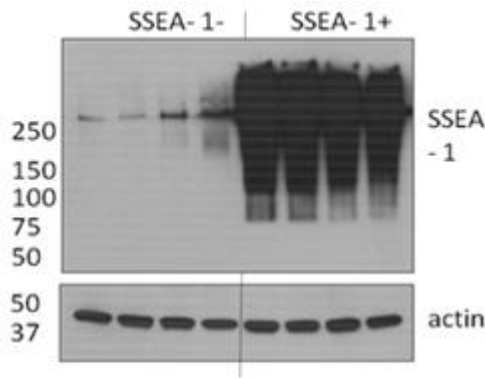
Figure 2.6 Western blot to confirm SSEA-1 enrichment and depletion in the respective fractions (provided by Anthony Valentijn, Liverpool Women's Hospital)
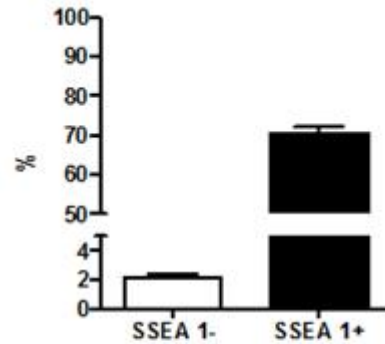
Figure 2.7 Efficiency of MACS separation as assessed by cytocentrifugation followed by immunofluorescence for SSEA-1 (provided by Anthony Valentijn, Liverpool Women's Hospital)

Fluorescence-Activated Cell Sorting (FACS)

With the use of cytocentrifugation and IF, our lab has seen that the cell purity and efficiency obtained by MACS for SSEA-1, is around 70% (figure 2.7) compared to FACS which has an efficiency of greater than 90%. For this reason, some samples were sorted on SSEA-1 and the epithelial marker CD9 using FACS, in order to verify the results obtained with MACS sorted samples. A limited number of samples could be sorted using FACS throughout the project due to the inaccessibility of the FACS instrument and the need for a high number of cells.

FACS instruments consist of a flow cytometer with the additional ability to sort cells according to their fluorescent signal. Fluorescently labelled single-cell suspensions flow past an excitation source (usually a laser light) of a single wavelength, in a liquid phase. The stream of cells is broken into individual droplets containing single cells. As they pass through the beam they cause the light to scatter, and this is analysed by detectors which can assess the cell structure (FC). In addition to this cell analysis, they can also be sorted with the use of electrostatic deflection which diverts cells into containers based upon their charge.

For FC, monolayer cultures of epithelial cells were trypsinised in to single cell suspensions as described for MACS (see section previous). Cells were labelled in FACS buffer (containing 0.5% **bovine serum albumin (BSA)** and 1mM EDTANa$_2$ in PBS) with either AlexaFluor 488-CD9, PE-SSEA-1 or both according to the manufacturer's instructions. Unlabelled cells and cells labelled with isotype matched antibodies served as controls. Cells were prepared and labelled by Anthony Valentijn and analysed and sorted by Stuart Marshall-Clarke or analysed alone by Sandra Rak-Razewska.

As with MACS, cultured epithelial cells which had been grown in monolayer were trypsinised in a 100mm culture dish by adding1ml 0.05% trypsin/EDTA solution and incubating at 37$^{\text{o}}$C for 5 minutes. 2ml of culture media containing FBS were then added to inhibit the trypsin. To break apart cell aggregates the solution was triturated and transferred through a 40μm cell strainer placed over a 50ml Falcon tube. Once centrifuged at 500g for 5 minutes at 4$^{\text{o}}$C the cell pellet was recovered. For FACS, at least 10^7-10^8 cells were required per test. Controls included unlabelled cells and cells which had been labelled with isotype controls for the fluorochromes which were used. The cell pellet was then resuspended in 100μl FACS buffer and between 0.5-1μg (dependent on the formulation of the manufacturer) of unlabelled or fluorescently labelled primary antibody for SSEA-1 and CD9, labelled with either Fluorescein (FITC)/AlexaFluorR 488 or Phycoerythrin (PE) as recommended by the manufacturers. This was then incubated in a dark fridge for 20-30 minutes. Following this, 1ml of FACS buffer was added and the tube was centrifuged at 700g for 3minutes at 4$^{\text{o}}$C. The supernatant was aspirated and the cell pellet was again resuspended in 1ml

of buffer before being centrifuged as before. In the case of the unlabelled primary antibody, the cell pellet was resuspended in 100μl FACS buffer and 0.5-1μg of the labelled secondary antibody was mixed in. This was incubated for 20-30 minutes at 4^oC and washed twice with 1ml of buffer. The unlabelled cells were then treated in the same way as cells mixed with the labelled antibody in the following step. If the labelled primary antibody was used, the cells were instead resuspended in 500μl FACS buffer and analysed as quickly as possible or fixed with **neutral buffered formalin (NBF)/PBS** and stored at 4^oC for up to one week.

Once the cells had been prepared as described above, they were analysed using the **FACSCalibur cytometer** along with Cell Quest Acquisition and Analysis software (CellQuestPro version). Cell sorting was performed using a FACS Ariall cell sorter and the data was finally analysed with FACSDiva software (version 6.1.3), BD Biosciences.

2.4 Organoid Culture

Mimicking the *in vivo* environment of the endometrial stem cell niche, endometrial epithelial cells were also cultured in 3D Matrigel, as well as the 2D culture previously described. Basement membranes are thin extracellular matrices that *in vivo* underlie cells. **BD Matrigel^{TM}** represents a solubilised form of basement membrane. Cells from MACS purification representing SSEA-1 enriched and depleted fractions were reuspended in Matrigel at an initial density of ~100,000 cells/100μl. This was subsequently diluted serially two-fold from ~100,000 cells/100μl to ~3000 cells /100μl. 50μl cells was plated in duplicate in wells of a 24-well tissue culture and the Matrigel allowed to gel at 37^oC for 30 min prior to adding 1.0ml DMEM/F12

supplemented with 1x **insulin-transferrin-selenium** and 50 ng/ml EGF. The medium was replaced every 3 days and the culture was monitored over a 10-12 day period. In 3D culture endometrial epithelial cells formed gland-like structures also referred to as organoids/spheroids similar to the glands seen in the endometrium. This mainly arose from the SSEA-1$^+$ cell population (figure 2.8).
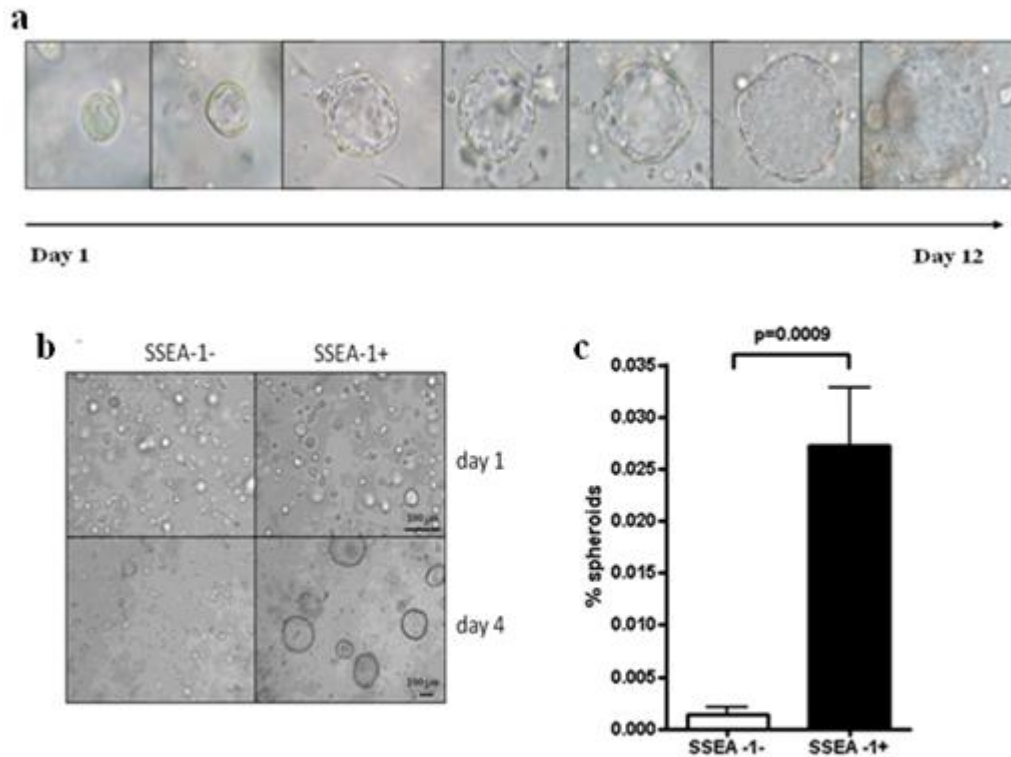
Figure 2.8 a) Morphological changes in spheroid structure over a 12 day period **b)** spheroids primarily arise from the SSEA-1 enriched cells **c)** the SSEA-1$^-$ fraction gives rise to significantly fewer spheroids (provided by Anthony Valentijn, Liverpool Women's Hospital)

2.5 RNA Extraction

2.5.1 RNA Extraction

In order to extract total RNA from SSEA-1 sorted cells and organoids, the TRIzol® reagent method was implemented according to the manufacturer's instructions. Initially, 1ml of **TRIzol® reagent** was added to the cell pellets in order to lyse the cells. In the case of positive controls and organoids which had been obtained from a

culture dish, culture medium was first aspirated and 1ml of TRIzol® reagent was then added directly to the dish. This was pipetted thoroughly to obtain a homogenous suspension before being transferred to a 1.5ml microcentrifuge tube. In order to ensure that the maximum numbers of cells were introduced into the TRIzol® suspension, cells were gently pipetted to ensure that the cells had visually disintegrated. In the instance that RNA had to be isolated from tissue samples, a **hand-held homogeniser** was used instead. Next, 200µl of **chloroform** (or $1/5^{th}$ of the initial volume of TRIzol®) was added to the solution. These microcentifuge tubes were then shaken for 15 seconds and centrifuged at $12,000g$ for 15 minutes at $4^{o}C$. At the end of this step, three phases could be distinguished. These included a clear upper aqueous layer which contained the RNA, a white interphase containing proteins which had not been fully denatured and a lower red organic phase made up of DNA and other proteins. The RNA or aqueous phase was then transferred into fresh 1.5ml tubes, ensuring that a clear margin was left above the interphase in order to minimise the risk of any contamination from DNA or protein. In cases where RNA was extracted from a small number of cells, the aqueous phase was added to separate tubes containing 1µg/µl of **glycogen**. The glycogen acts as a carrier and is useful when small amounts of RNA are extracted. The remaining microcentifuge tubes containing the lower two phases were discarded at this point into 'TRIzol waste'. In order to precipitate the RNAs, 500µl of **2-propanol** (50% of the original volume of TRIzol® used) was added and the solution mixed by inverting the tubes 6 times. These were then left to incubate for 10 minutes at room temperature. Subsequent to incubation, the tubes were centrifuged at $12,000g$ for 10 minutes at $4^{o}C$ to produce visible RNA pellets at the bottom of the tubes. The supernatant was removed and the remaining pellet was washed with 1ml of 75% **ethanol** and was centrifuged at $7,500g$ for 5 minutes at $4^{o}C$. The ethanol was

then discarded, and the RNA pellets left to dry for a few minutes at room temperature. As the RNA was then used immediately for cDNA synthesis, the pellets were dissolved in 15-25µl **nuclease free water** depending on their size.

2.5.2 Verification of RNA Integrity by Gel Electrophoresis

RNA integrity was verified with the use of electrophoresis on a 1% agarose gel (made in 1x tris–acetate–ethylenediamine tetraacetic acid (TAE)). This was achieved by initially dissolving 2g of **agarose** power in 200ml of 1x TAE buffer (recipe in table

Table 5: Recipe for 10x TAE

Recipe for 10x TAE	
NaCl	48.4g
Glacial Acetic Acid (17.M)	11.4ml
EDTA	3.7g
Distilled H_2O	Up to 1L

3). This suspension was heated to boiling point in a microwave before allowing it to cool to 60°C. 4µl of **ethidium bromide** (2µl per 100ml; 0.5µl/ml) was then added and mixed into the molten agarose before pouring it into a plastic electrophoresis case of the appropriate size. Ethidium bromide staining is used to enable visualisation of the 28S and 18S ribosomal RNA bands. The gel was then left to set for around 30 minutes at room temperature. Samples to be loaded were prepared during this time included 1µl of RNA, 2µl of **5x DNA loading buffer** and 7µl of nuclease-free water, making a final volume of 10µl. The 5x loading buffer allowed visual control of the electrophoresis and provided density to the samples. It was added to each sample to a 1x final concentration. The gel was then immersed in the 1x TAE buffer and a 100V electrical current was applied. RNA of good quality appeared as two clear rRNA bands (28S and 18S) and in most cases samples showed a third band nearer the end of the gel representing 5S. Degraded RNA appeared on the gel as a smear. Bands were observed

under a UV light in a ***Molecular Imager® Gel DocTM XR*** from Bio-Rad.

Representative RNA Bands for SSEA-1 sorted samples are shown below (figure 2.8):

Normal Endometrial Samples (labelled 'N')**:**

1. MACS Sorted
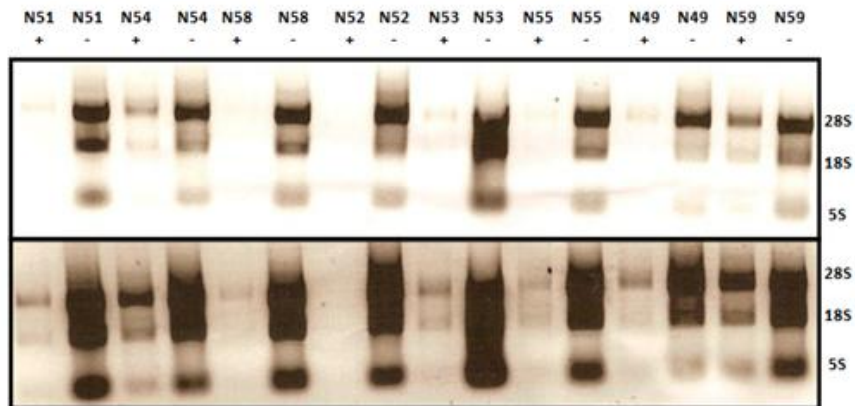
Figure 2.9 Sample RNA bands; + = SSEA-1$^+$ fraction; - = SSEA-1$^-$ fraction. Note: both gels (top and bottom) show the RNA bands for the same samples, however the lower gel has been overexposed in order to visualise fainter bands.

2. FACS Sorted

No clear bands seen
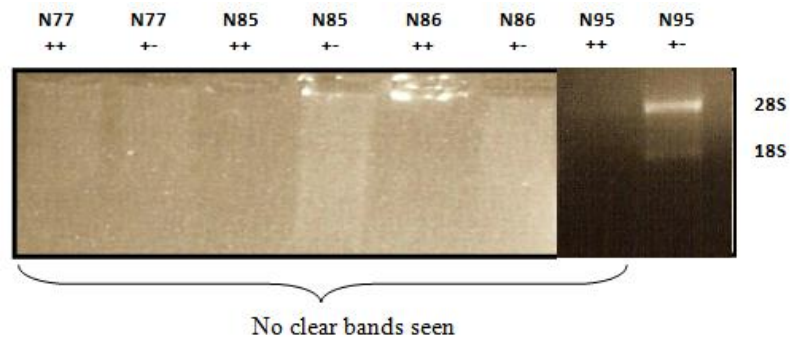
Figure 3.0 Sample RNA bands; ++ = CD9$^+$SSEA-1$^+$; +- = CD9$^+$SSEA-1$^-$

Endometriosis Samples (MACS only) (labelled 'E'):
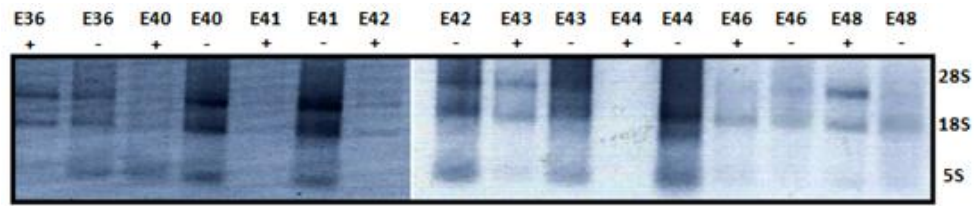
Figure 3.1 Sample RNA bands; + = SSEA-1$^+$ fraction; - = SSEA-1$^-$ fraction

Organoids (MACS only):
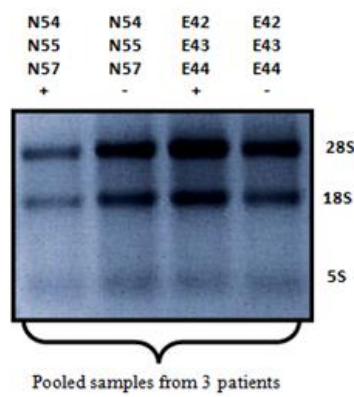
Figure 3.2 Sample RNA bands; + = SSEA-1$^+$ fraction; - = SSEA-1$^-$ fraction

2.5.3 RNA Quantification: NanoDrop

In order to determine the RNA concentration within each sample, RNA samples were loaded onto a **NanoDrop**^{TM} **1000 Spectrophotometer** following the manufacturer's guidelines. Firstly, the **Nanodrop 2000 software** was loaded and the machine was verified with the arm of the Nanodrop facing downwards. 1µl nuclease-free water was then loaded to blank the machine. This nuclease-free water should be the same as that used to dissolve the initial RNA pellet. Following this, 1µl of the RNA samples were loaded in succession and the RNA concentrations measured. The 260/280nm absorbance ratio, reflecting the purity of the RNA, was also measured. Pure samples have values between 1.8 and 2. Quantification of the RNA at this stage allowed equalisation of the RNA input within the next stage of DNase treatment. On

completion of use, the NanoDrop was cleaned with 1μl nuclease-free water (see appendix VII for nanodrop values).

2.6 cDNA Synthesis

2.6.1 DNase Treatment

In order to remove any contaminating genomic DNA from the RNA samples, the RNA was subsequently treated with deoxyribonuclease (DNase) enzyme. Dependent on previous Nanodrop readings, the volume of RNA was altered accordingly in order to equalise the RNA concentrations which were input into the DNase treatment step. For each sample a maximum of 8μl of RNA (typically a total of approximately 1000-1500ng), 1μl **RQ1 DNase Buffer** and 1μl **RQ1 DNaseI (1000U/ml)** were placed into a **0.2ml PCR tube** and incubated for 30 minutes at 37°C. Following this, 1μl of **RQ1 DNase Stop Solution** was added and incubated for a further 15 minutes at the higher temperature of 60°C in order to completely inactivate the DNase. The DNase-treated RNA was then used immediately for cDNA synthesis and the remaining volume was stored at -20°C until it was next required.

2.6.2 cDNA Synthesis

For cDNA synthesis, 4μl of the DNase-treated RNA, 2μl of **random hexamers** (100ng/μl), 1μl of **dNTP mix** and 7μl of nuclease-free water were added into a fresh 0.2ml tube. This was incubated at 65°C for 5 minutes and then placed on ice for 1 minute. Next, 4μl of **5x 1st strand buffer**, 1μl of **dithiothreitol (DTT) (0.1M)** and 1μl of the reverse transcriptase enzyme **SuperScript III (200U/μl)** were added to the tube, mixed gently and left to stand at room temperature for 5 minutes. Finally this was incubated for 60 minutes at 50°C followed by a further 15 minutes at 70°C in order to

inactivate the enzyme. The cDNA was diluted 1:3 (v/v) with nuclease-free water before being stored at -20°C until further use.

2.7 Real-Time Polymerase Chain Reaction

2.7.1 Primers

All of the primers used within this study were purchased from Sigma-Aldrich in lyophilised form. The primers were dissolved in 1ml of nuclease-free water to make a concentrated stock, which was then diluted to make working stocks of 6.25pmol/µl concentration. Primers which were designed in house were sequenced by the Sequencing Service, University Dundee, UK. Details about the primers used within this study are included in table 6.

Table 6 Human specific primer pairs

Gene	Sequence	Product Size (bp)	Annealing Temperature (°C)	Source
OCT4	F: 5'AGAACCGAGTGAGAGGCAA3' R: 5'CTCTCGTTGTGCATAGTCGC3'	176	56	In house
NANOG	F: 5'CGGAGACTGTCTCTCCTCTT3' R: 5'GTTCTTGCATCTGCTGGAGG3'	240	56	In house
SOX2	F: 5'CGAGATAAACATGGCAATCAAAAT3' R: 5'AATTCAGCAAGAAGCCTCTCCTT3'	85	56	Wong *et al*, 2010
PODXL	F: 5'CCATCGTCTGCATGGCATCA3' R: 5'CTGTCTGCAGCTCCTCTGTT3'	114	56	In house
CD133	F: 5'TGCAACAGCATCAGATTGTC3' R: 5'TACCTGCTACGACAGTCGTG3'	199	56	In house
CD9	F: 5'GACACCTACAACAAGCTGAA3' R: 5'ACAGGACTTCACGGTGAAGG3'	165	56	In house
ERα	F: 5'TGATTGGTCTCGTCTGGCG3' R: 5'CATGCCCTCTACACATTTTCCC3'	101	56	Henderson, *et al* 2003
PR	F: 5'CAGTGGGCGTTCCAAATGA3' R: 5'TGGTGGAATCAACTGTATGTCTTGA3'	83	56	Henderson *et al*. 2003
FUT4	F: 5'CAGCTGGTTCGAGCGGTGAAGCCGCGCT3' R: 5'CAGAAAAACGTGAATCGGGAACAGTTGTGT3'	435	60	Ponnampalam *et al*, 2008
ACTB	F: 5'AGTGTGACGTGGACATCCGCA3' R: 5'GCCAGGGCAGTGATCTCCTTCT3'	112	56	Marullo *et al*, 2010
GAPDH	F: 5'GTGGTCTCCTCTGACTTCAA3' R: 5'TCTCTTCCTCTTGTGCTCTT3'	212	56	In house
YWHAZ	F: 5'CGTTACTTGGCTGAGGTTGCC3' R: 5'GTATGCTTGTTGTGACTGATCGAC3'	69	56	Marullo *et al*, 2010

2.7.2 Real-Time/Quantitative PCR (qPCR)

In order to quantify the level of mRNA expression for the specific genes identified in the table above, real-time qPCR was performed using SYBR green in a 20µl PCR reaction: 10µl **KAPA SYBR FAST qPCR Mix Master (2x)**, 1µl forward primer (6.25pmol/µl or 0.3125µM), 1µl reverse primer (6.25pmol/µl or 0.3125µM), 1µl cDNA template and 7µl nuclease-free water. qPCR allows both the detection and quantification of the amount of amplified double stranded DNA by measuring the level of fluorescence. SYBR green is a non-specific fluorescent dye which intercalates with double-stranded DNA only, emitting a fluorescent signal of a specific wavelength on binding. The intensity of the signal is therefore relative to the number of copies amplified, and increases with increasing cycle number. To minimise the variations made when pipetting, general primer and template master mixes were prepared before making up the 20µl PCR reactions. For each PCR reaction, 11µl of the template master mix (containing the KAPA SYBR FAST qPCR Mix Master (2x) and template) and 9µl of the primer master mix (containing the specific forward and reverse primers and nuclease-free water) were mixed in a 0.2ml PCR tube, to make a final volume of 20µl. qPCR reactions were performed in triplicate for each template using the **Corbett Rotor-Gene 3000 centrifugal real-time cycler** (36 well rotor) along with Rotor Gene Software (Version 6) according to the following cycling conditions: 95°C for 10 minutes to activate the DNA polymerase, followed by 35-40 cycles of denaturation at 95°C for 6 seconds, annealing at 55-60°C for 20 seconds and elongation at 72°C for 30 seconds. For generation of a melt curve within the final stage of each run, the temperature would ramp from 72°C up to 95°C, rising by 1°C with each step. 'No template' reactions were included with each PCR run as a negative control, in order to

identify the presence of any contamination. Positive controls were also included to ensure that the specificity of the primers (listed in table 7).

Table 7 Primer positive controls

Positive Control	Primers
Full thickness endometrium (Proliferative phase)	*ERα, PR, PODXL, CD9, GAPDH, ACTB, YWHAZ*
Human ESCs: Hues 7, passage 28	*OCT4, NANOG, SOX2*
HT29	*CD133,*
Secretory Phase Endometrium	*FUT4*

The amplification products were initially verified using gel electrophoresis to confirm the correct band sizes and hence the correct amplicon. For further experiments, the melt curve generated by the software with each run, gave sufficient evidence of the correct product and also additional information of any non-specific amplification or primer dimers. In order to calculate relative mRNA expression, all genes were compared to the reference gene *YWHAZ* (Marullo *et al*, 2010), which was included within each run.
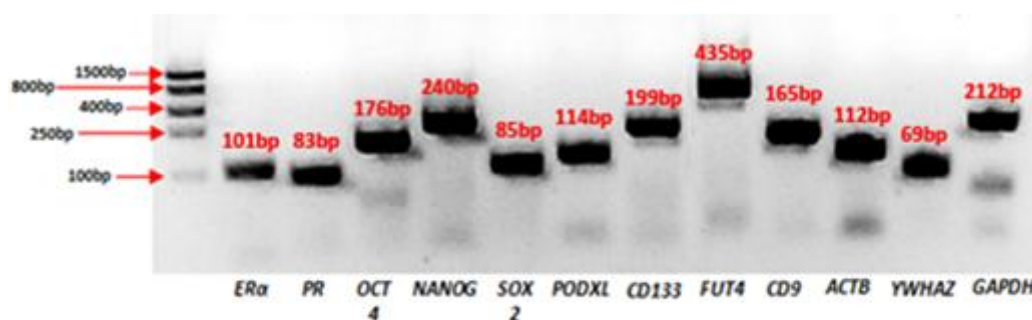
Figure 3.3 Gel electrophoresis to confirm PCR product sizes. Primers were checked against their positive controls

2.7.3 Verification of cDNA Synthesis

In order to verify that the cDNA synthesis protocol worked correctly, the template was tested using one qPCR experiment. The templates used in this run were RNA,

DNase treated RNA and cDNA for a particular sample, in addition to the no-template control (NTC). For this run only, PCR reactions were not performed in triplicate but as single reactions using the primers for the reference gene alone. If the DNase treatment is successful, RNA should show some amplification due to contaminating genomic DNA; however, this should be eliminated following DNase treatment. High amounts of amplification should be evident when using cDNA as template.

Quantification Analysis
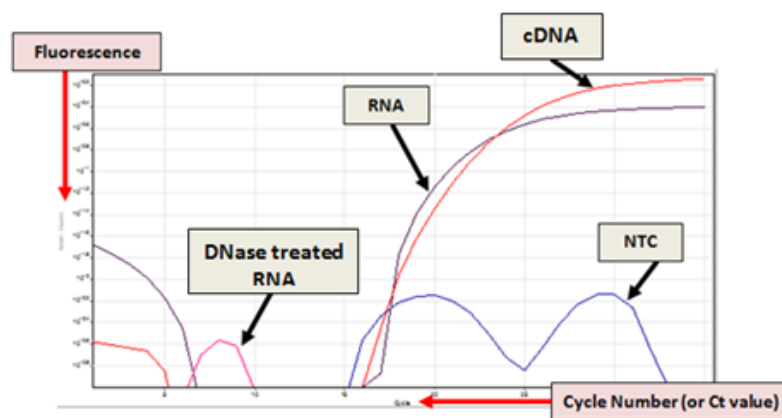
Figure 3.4 Representative amplification profile to show verification of DNase treatment and cDNA synthesis protocol using qPCR. Template= full thickness endometrium; primer= GAPDH

Melt Curve Analysis:
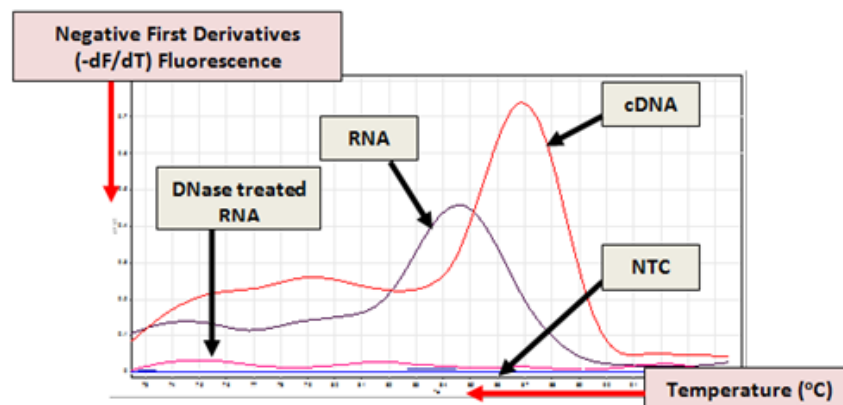
Figure 3.5 Representative melt curve to show verification of DNase treatment and cDNA synthesis protocol using qPCR. Melting point reflects GC content of amplicon and therefore product size. Template= full thickness endometrium; primer= GAPDH

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2.7.4 Efficiency of Primers

In order to calculate the relative gene expression, the efficiency of each primer set had to be determined using standard curves to assess the linearity of DNA amplification. To achieve this, serial dilutions of cDNA template from a positive control were used to perform a qPCR run for each primer set. Serial dilutions of the cDNA diluted in nuclease-free water were made up according to the following diagram (figure 3.6).
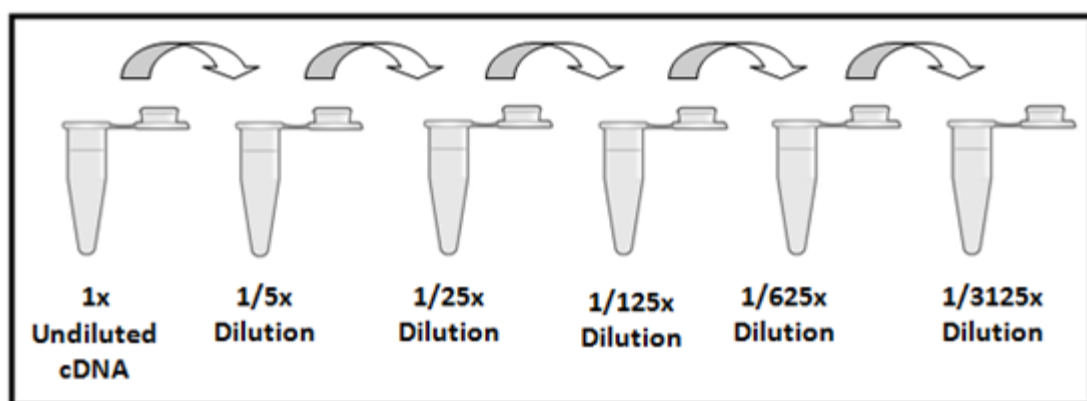
Figure 3.6 Preparing a serial dilution

Note that the 'undiluted cDNA' was diluted 1:3 (v/v) with nuclease free water (as described within the cDNA synthesis section 2.6), and this was considered to be the 1x sample. The remaining dilutions were made up by diluting 1:5 (v/v) from the previous dilution. qPCR reactions were set up as described previously using three technical replicates. This generated a standard curve for each primer set which reported their efficiency. The efficiency of a primer is dependent on the amount of specific PCR product which is yielded at the end of each cycle. Ideally a primer which is 100% efficient should produce a 2-fold increase in PCR product with each cycle. This is denoted as an efficiency of 1 on the Rotor-Gene 6 software. A representative standard curve for *NANOG* has been shown below (see appendix VIII for all standard curves).
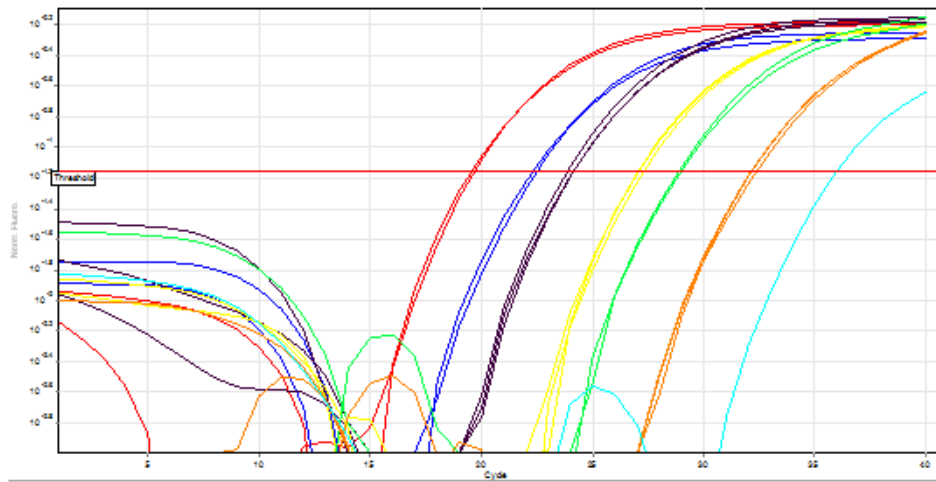
Figure 3.7 Representative standard curve for NANOG: 1x *NANOG* (red), 1/5x *NANOG* (blue), 1/25x *NANOG* (purple), 1/125x *NANOG* (yellow), 1/625x *NANOG* (green), 1/3125x *NANOG* (orange), NTC (turquoise). Positive control template= ESCs Hues7 P28
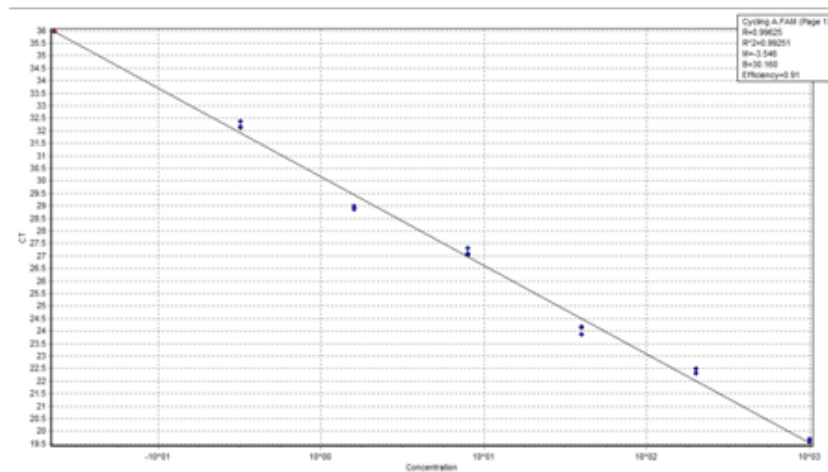
Figure 3.8 Graph to represent *NANOG* primer pair efficiency. Efficiency= 0.91

2.7.5 Calculating Relative Expression

For each standard curve, sample dilutions were given arbitrary numbers of copies ranging from 0.32 up to 1000, as shown in table 8 below.

Table 8 Theoretical concentrations for serial dilutions

Template Dilution	Given Concentration (copies/µl)
1x	1,000.00
1/5x	200.00
1/25x	40.00
1/125x	8.00
1/625x	1.60
1/3125x	0.32

Following each qPCR run, thresholds were set at the exponential phase of the amplification profile. Ideally this would be set where the regression correlation coefficient (R^2) is optimal, however to allow comparison between all genes and the standard curves across all samples, the threshold was set to a fixed value of **0.07**. Using this threshold, the cycle number (Ct) values, indicating the number of thermal cycles needed for the fluorescent signal in a given sample to reach the threshold, could be deduced. Using the standard curves, the reaction efficiency based on the efficiency of the primer pairs could be calculated using the formula **Efficiency*=10$^{(-1/m)}$ – 1**, where m represents the gradient of the regression line. The theoretical/arbitrary copy numbers allocated to the varying dilutions was plotted against the Ct values to form a standard curve for each primer pair. The equations of these curves were used within qPCR mRNA analysis on the target samples, in order to calculate the relative number of copies of mRNA within them (see table 9).

Table 9 Standard curve concentration equations

Primer Set	Melting Point (^{o}C)	Efficiency	Standard Curve Concentration Equation (Conc=)
OCT4	87.8	0.95	$10^{(-0.289*Ct + 10.868)}$
NANOG	84.3	0.91	$10^{(-0.282*Ct + 8.505)}$
SOX2	77.5	0.73	$10^{(-0.245*Ct + 9.238)}$
PODXL	87.3	1.02	$10^{(-0.306*Ct + 11.058)}$
CD133	83.8	1.01	$10^{(-0.303*Ct + 9.615)}$
FUT4	85.8	0.74	$10^{(-0.240*Ct + 9.989)}$
PR	80.7	1.08	$10^{(-0.318*Ct + 11.543)}$
ERα	83.5	1.01	$10^{(-0.304*Ct + 11.066)}$
CD9	85.7	1.26	$10^{(-0.356*Ct + 12.876)}$
ACTB	86.7	0.86	$10^{(-0.269*Ct + 8.418)}$
YWHAZ	81.0	1.14	$10^{(-0.303*Ct +9.615)}$

2.7.6 Gel Electrophoresis: FlashGel System

In order to confirm that the primer sets were amplifying the specific PCR products, gel electrophoresis was used in order to check that these products were of the correct band sizes (as mentioned in section 2.7.2). Unlike the system used to check RNA bands with self-made agarose gels (section 2.5.2), the PCR products were verified using the FlashGel system from Lonza. This system is the fastest way to separate DNA using disposable, pre-cast and pre-stained agarose gel cassettes whilst allowing real time monitoring of DNA migration. The FlashGel system is not
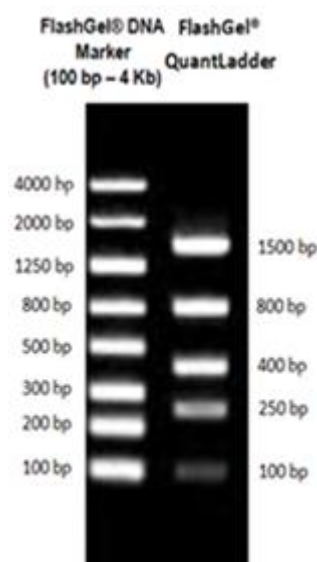
Figure 3.9 FlashGel® DNA markers

only advantageous in terms of time; however it is also a more sensitive and safer method, eliminating exposure to dangerous chemicals such as ethidium bromide and

also UV illumination. As special RNA cassettes were needed, this system was not ideal for visualising RNA bands which appear as a smear on the DNA cassettes. Therefore the previously described method of gel electrophoresis was used for this.

The Flashgel system was used according to the manufacturer's instructions. Samples to be loaded were first prepared on **parafilm**, including 1µl of PCR product, 1µl 1x **FlashGel™ Loading Dye (5x)** and 3µl nuclease-free water to make up a maximum final sample volume of 5µl per well. Next, the white well seal was removed from either the **two-tier 16+1 2.2% agarose cassette** whilst taking care not to remove the clear side vents. Sample wells were then flooded with distilled water and by tilting the cassette, excess fluid which had moved to the edge of the cassette could be blotted away using tissue paper. The wells were not blotted directly. Following this, the cassette was placed into the **FlashGel dock** and the samples were loaded, avoiding the first well. The first well was loaded with 3µl of the **FlashGel® DNA Marker 100bp-4kb** or the **FlashGel® Quantladder (100bp-1.5kb)**. Next, the voltage leads were assembled and the power supply and light were both turned on. The voltage was set to 270V and the gel was left to run for between 2-5 minutes until the desired separation had been achieved. When completed, the power was then switch off and the leads disconnected. The bands were then recorded and an image captured using the **FlashGel™ Camera**.

2.8 Immunofluorescence

IF works by using the specificity of antibodies to detect target antigens within a cell. This binding can by visualised with the use of fluorescent dyes which emit light of different wavelengths following the absorption of excitation light. There are two main methods of IF labelling; direct and indirect labelling. For the purpose of this

study, indirect labelling was used involving an unlabelled primary antibody which was specific to the molecule of interest. A secondary antibody, tagged with a fluorescent dye, was then targeted towards the
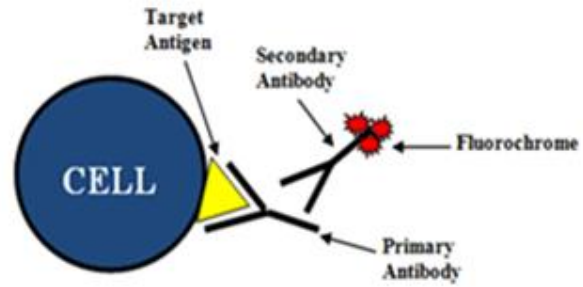
Figure 4.0 Schematic drawing of indirect IF

constant portion of the first antibody (figure 4.1). Unlike IHC, in our study IF allowed dual labelling of the protein of interest and cytokeratin (*CK*), a membrane protein found within the cytoskeleton of epithelial cells. This allowed the exclusion of any staining that may have arisen from other contaminating cell types.

Due to time restraints, this was performed on one sample taken from a healthy female of reproductive age with no gynaecological pathology. Following MACS sorting (described in section 2.3.2), the endometrial SSEA-1$^+$ and SSEA-1$^-$ epithelial cells were plated onto two separate **8-well chamber slides** at a seeding density of approximately 5×10^3 cells/well (dependant on the yield of SSEA-1$^+$ cells). Following this the cells were left to adhere and proliferate within the wells for approximately 2 days before fixing. In order to fix the cells, they were washed twice with 1x PBS. Each was done for 5 minutes at room temperature. 10% NBF was then added for 10 minutes at room temperature and the chambers were subsequently washed three times with 1x PBS before proceeding onto the staining protocol.

In order to permeabilise the cell membranes, the PBS was aspirated and the wells were covered 0.2% triton/EDTA and incubated for 5 minutes at room temperature. Following this the wells were washed once with PBS and incubated with blocking

serum for one hour at room temperature in order to block non-specific sites. The block/diluent was prepared with1% BSA, 10% **normal goat serum (NGS),** 0.05% **tween** and PBS within a **100ml universal tube.** Within this time appropriate dilutions of the primary antibodies and *CK* (for dual labelling) were prepared according to the concentrations indicated in the table and the plan of the 8-well chamber slides below. These were diluted with the diluent previously prepared (table 10). Following the blocking stage, 200µl of each the appropriate antibody was applied to each well and incubated at 4°C overnight. Note that block was left on one well per chamber which served as a negative control with no primary antibody (see figure 4.2).

Table 10 Detailed specifications of primary antibodies

Primary Antibody	Supplier	Catalogue Number	Clone	Concentration	Polymer	Mono/ polyclonal
OCT4A	Cell Signalling Technology	C30A3	C30A3	1:50	Rabbit (Rb)	Monoclonal
NANOG	Cell Signalling Technology	D7364	D73G4	1:100	Rabbit (Rb)	Monoclonal
PODXL	R&D Systems	MAB1658	222328	1:100	Mouse (Ms)	Monoclonal
Telomerase	Abcam	ab27573		1:500	Rabbit (Rb)	Polyclonal
ERα	Epitomics	S1353		1:100	Rabbit (Rb)	Polyclonal
ERβ	Serotec	MCA19745	PPG5/10	1:50	Mouse (Ms)	Monoclonal
PR	Dako	M3569	PgR636	1:200	Mouse (Ms)	Monoclonal
CK18	Dako	M7010	DC10	1:200	Mouse (Ms)	Monoclonal
CK7	Cell Signalling Technology	4898S	R458	1:200	Rabbit (Rb)	Polyclonal

SSEA-1⁺ 8-Well Chamber Slide		SSEA-1⁻ 8-Well Chamber Slide		Secondary Antibody
OCT4 (Rb) &CK18 (Ms)	*ERα (Rb) &CK18 (Ms)*	*OCT4 (Rb) &CK18 (Ms)*	*ERα (Rb) &CK18 (Ms)*	*Alexa Fluor® 555 (anti-rabbit) & Alexa Fluor® 488 (anti-mouse)*
NANOG (Rb) &CK18 (Ms)	*Telomerase (Rb) &CK18 (Ms)*	*NANOG (Rb) &CK18 (Ms)*	*Telomerase (Rb) &CK18 (Ms)*	
ERβ (Ms) &CK7 (Rb)	*PODXL (Ms) &CK7 (Rb)*	*ERβ (Ms) &CK7 (Rb)*	*PODXL (Ms) &CK7 (Rb)*	*Alexa Fluor® 488 (anti-rabbit) & Alexa Fluor® 555 (anti-mouse)*
Negative Control (No primary antibody)	*PR (Ms) &CK7 (Rb)*	*Negative Control (No primary antibody)*	*PR (Ms) &CK7 (Rb)*	

Figure 4.1 Primary and secondary antibody staining plan for 8-weel chamber slides

Following overnight incubation, the wells were washed three times with PBS and 200µl of the secondary antibody was added to each well. The secondary antibodies were diluted to a final concentration of 1:1000. These antibodies were chosen to ensure that all *CK* was stained green and that the target protein was stained red. The top four wells of each chamber was incubated with anti-rabbit Alexa Fluor® 555 (red) and anti-mouse Alexa Fluor® 488 (green), and the bottom four wells were stained with anti-rabbit Alexa Fluor® 488 Conjugate (green) and anti-mouse Alexa Fluor® 555 (red) (as indicated in figure 4.2).

Table 11 Detailed specifications of secondary antibodies

Secondary Antibody	*Supplier*	*Catalogue Number*	*Concentration*	*Species*	*Polymer*
Alexa Fluor® 488 Conjugate (green)	Cell Signalling	4408	1:1000	Goat	Anti-mouse
Alexa Fluor® 555 Conjugate (red)	Cell Signalling	4409	1:1000	Goat	Anti-mouse
Alexa Fluor® 488 Conjugate (green)	Cell Signalling	4412	1:1000	Goat	Anti-rabbit
Alexa Fluor® 555 Conjugate (red)	Cell Signalling	4413	1:1000	Goat	Anti-rabbit

Details of the secondary antibodies can be seen in the table 11 above. The chambers were then left to incubate at room temperature in a dark cupboard for 45 minutes,

allowing time for the secondary antibodies to bind. The chambers were subsequently washed with PBS three times and using the special **removal tool** the glass chamber was separated from the slide below. The slides were quickly dipped into water to maintain moisture before adding one drop of **mounting medium with DAPI** onto each well. The DAPI allowed visualisation of cell nuclei with blue staining. Carefully, one rectangular **22x40mm coverslip** was placed on each slide so that all wells would be covered and any bubbles were removed. The slides were then visualised using a fluorescence microscope and images captured using NIS Elements-F software. MacBiophotonics ImageJ software was used to overlay the images taken of the different fluorophores. IF staining of passage 28 Hues7 humam embryonic stem cells (provided by Virginie Mournetal, Liverpool Stem Cell Group) were also included as a positive control in order to verify the staining shown by the *OCT4A* and *NANOG* antibodies.

2.9 Statistical Analysis

Data was considered to be non-normally distributed as recommended by a professional statistician and confirmed by SPSS normality testing using the Shapiro-Wilk's test (p-value less than $\alpha=0.05$), therefore non-parametric methods of data analysis were used. Statistical analysis was performed using the non-parametric paired Wilcoxon signed-rank test (WSR test), in order to compare the differences in gene expression between the SSEA-1$^+$ and SSEA-1$^-$ fractions, and also across the normal and endometriosis groups. The Mann-Whitney U-test (MWU test) for nonparametric independent groups was used to check for baseline differences in patient demographics between the normal and endometriosis groups. Summary measures in the form of medians and interquartile ranges (IQRs) were also used for

the analysis of non-parametric data. The software package GraphPad Prism 5 was used aid with data analysis. P<0.05 was considered as a statistically significant result.

```
                          ┌─────────────────────────┐
                          │    Sample Collection    │
                          └─────────────────────────┘
                                      │
                                      ▼
                    ┌──────────────────────────────────────┐
                    │ Epithelial cell separation and enrichment │
                    └──────────────────────────────────────┘
                         │                        │
                         ▼                        ▼
                ┌──────────────┐          ┌──────────────┐
                │ MACS Sorting │          │ FACS Sorting │
                └──────────────┘          └──────────────┘
```

3D cell culture
10-12 days of cell cultivation

(Healthy endometrium n=3 pooled;
Endometriosis n=3 pooled)

2D cell culture
Up to 3 days of cell cultivation

(Healthy endometrium n=7;
Endometriosis n=7)

RNA extraction and cDNA synthesis

Immunofluorscence
(n=1)

*q*PCR

Statistical analysis
(For non-parametric data)

Figure 4.2 Flowchart to summarise the materials and methods

Chapter 3

Validation of Primers for use in qPCR

Chapter 3: Validation of Primers for use in qPCR

3.1 Validating the Reference Gene

Within each well, a single qPCR experiment measures the intensity of Sybr green fluorescence, which is proportionate to the amount of PCR product, and thus indicates gene expression levels within a certain sample, under specific experimental conditions. In order to increase the reliability of any relative qPCR experiment, an important step in gene expression analysis is the normalisation of the data to a reference gene. Normalisation helps to correct for any technical variation which may occur due to pipetting differences and also corrects for sample to sample variation in qPCR efficiency. The most frequently used approach is to normalise the mRNA level to an internal standard, also referred to as a reference or housekeeping gene which is assumed to display an equal level of expression in all cells within the control or experimental conditions under investigation. To achieve accurate quantification of mRNA levels, a suitable reference gene must be chosen which does not vary between the control and experimental conditions.

Along with beta actin (*ACTB*) and *18s ribosomal RNA* (*18s* rRNA), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is conventionally used and widely accepted as a housekeeping gene. Its role is vital within carbohydrate metabolism as this gene codes for the enzyme required to catalyse the sixth step of glycolysis (Selwood T, 2012). *GAPDH* is commonly used without validation of its suitability as a reference, and therefore it was for this reason that *GAPDH* was initially used whilst optimising the qPCR experiments within this study. With time, as the number of qPCR experiments that were being performed within this study increased, contamination within the *GAPDH* NTC appeared and gradually increased. Although individual components of

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the qPCR experiment such as the nuclease-free water, the Taq polymerase and the *GAPDH* primer stock were changed, the contamination could not be eliminated, making the results unacceptable. This meant that an alternative reference gene had to be located before progressing to assess gene expression within the target samples.

On exploration of the literature it was quickly discovered that although commonly used, *GAPDH* is no longer regarded as the most stable housekeeping gene and has been shown to vary considerably between different samples and tissue types (de Leeuw *et al*, 1989). It is likely that the changing physiological processes that occur throughout the menstrual cycle and the altered tissue environments within different gynaecological pathologies such as endometrial cancer or endometriosis, also have an effect on the expression levels of the housekeeping genes. Studies investigating placental gene expression at various stages of pregnancy have shown variable expression of the reference genes *GAPDH* and *ACTB* (Patel *et al*, 2002; Meller *et al*, 2005). In addition, *18s* and *ACTB* have been shown to display significant variation throughout the menstrual cycle (Sahlin, 1995; Ejskjaer et al, 2009). These studies led to further investigation and comparison of the stability of a range of housekeeping genes within the endometrium using GeNorm, a system used to select the best candidate reference gene. Sadek *et al* compared the stability of these genes within healthy endometrial tissue and tissue from women suffering from polycystic ovarian syndrome (PCOS) and found that out of nine reference genes, only *YWHAZ*, *CYC1* and *ACTB* were stable within the experimental conditions. *GAPDH* was ranked as the worst housekeeping gene (Sadek *et al*, 2012). Vestergaard *et al* also explored the stability of housekeeping genes within eutopic and ectopic endometrium collected from women with endometriosis and healthy women. It was found that out of seven

reference genes, *TBP* and *YWHAZ* were ranked the best pair using GeNorm analysis and that *YWHAZ* was the single most stable gene using NormFinder analysis (Vestergaard *et al*, 2011). It was following this literature search that *YWHAZ* was considered as a suitable housekeeping gene to explore and test under our experimental conditions. Tyrosine 3-monooxygenase also known as *YWHAZ*, is a gene which belongs to the 14-3-3 family of proteins and acts to mediate cell-cell signal transduction by binding to phosphoserine-containing proteins (NCBI, 2012).

In order to confirm that *YWHAZ* would be a reliable housekeeping gene, its variability was compared to the more widely accepted and accredited *ACTB* on some of the normal endometrial samples which were collected for this project. Its stability was also investigated on endometrial tissue collected from a female with endometriosis to assess whether it was stable within the target pathology within this study. The histogram in figure 4.3 shows the cycle differences or ΔCt values between *YWHAZ* and *ACTB* when their gene expression was measured within the normal SSEA-1 MACS sorted endometrial samples, collected for the purpose of this study. It can be seen here that there was minimal variation in the ΔCt values between *ACTB* and *YWHAZ* (average ΔCt value of 4.34), proving the stability of *YWHAZ* amongst the study samples (figure 4.3).

The following quantification curve illustrates that *YWHAZ* does not vary in patients with endometriosis also when compared to *ACTB* (figure 4.4).
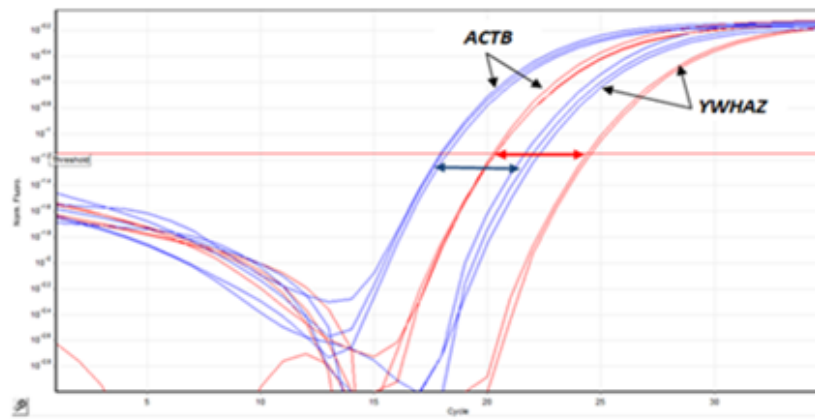
Figure 4.4 Quantification curve to show that cycle differences between *ACTB* and *YWHAZ* do not vary between normal endometrial tissue (red amplification curves) and endometriosis tissue (blue amplification curves). Approximately 4 cycles difference as a threshold of 0.07

The cycle difference between *YWHAZ* and *ACTB* was found to be 4.18 cycles within full thickness normal endometrial tissue (represented by the red arrow) at a threshold of 0.07. Within eutopic endometrial tissue taken from a patient with endometriosis the cycle difference between *ACTB* and *YWHAZ* was found to be 3.92 cycles at the same threshold. These results prove that there was around a 4 cycle difference in gene expression between the two reference genes, and this did not vary within endometriosis. It could therefore be concluded that the expression of *YWHAZ* is stable within endometriosis.
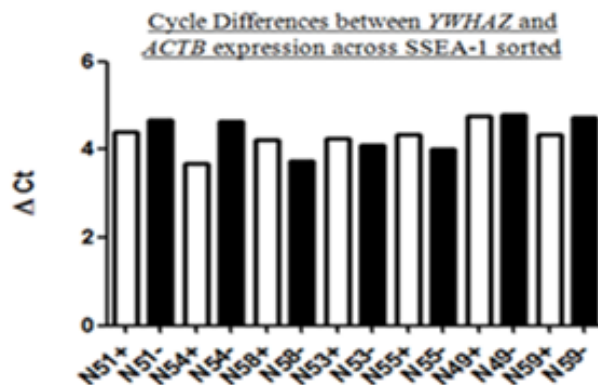
Figure 4.3 Graph to show the average ΔCt values between *YWHAZ* and *ACTB* in 7 normal SSEA-1 MACS sorted endometrial samples

3.2 Validation of Specific Primers

3.2.1 human telomerase reverse transcriptase (*hTERT*)

hTERT was initially included as one of the target genes to study within the SSEA-1 sorted cells. However, it quickly became evident that *hTERT* mRNA was present at very low levels within the sorted cells and generating quantifiable results via qPCR proved extremely difficult. Three different primer sequences for human specific *TERT* were already available within the lab. The sequences for these are shown below (table 12):

Table 12 *hTERT* primer pair sequences (Pairs 1-3)

Gene	Sequence	Product Size	Reference
TERT 1	F: 5'CCGCCTGAGCTGTACTTTGT3' R: 5'CAGGTGAGCCACGAACTGT3'	234	Rahmati-Yamchi M, 2011
TERT 2	F: 5'AGGGGCAAGTCCTACGTCCAGT3' R:5'CACCAACAAGAAATCATCCACC3'	159	Meeran SM, 2010
TERT 3	F: 5'CGTACAGGTTTCACGCATGTG3' R: 5'ATGACGCGCAGGAAAAATG3'	82	Lehner R, 2002

To select the best primer pair, qPCR was performed using all three primer sets on a range of different positive control templates, including endometrial cancer, Hues7 ESCs and full thickness endometrium taken during the proliferative phase. The amplification products were run on an agarose gel and the following results were generated (figure 4.5).
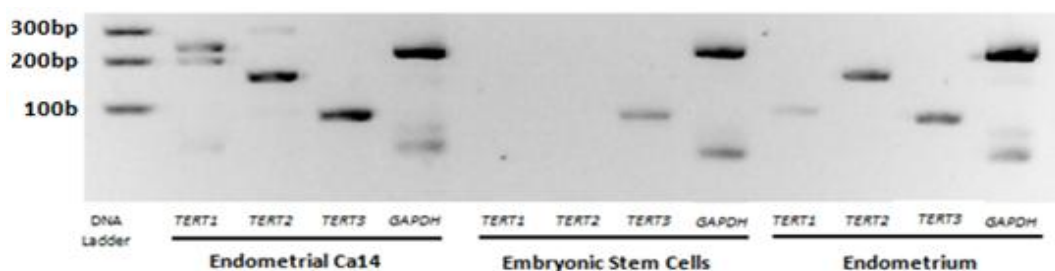
Figure 4.5 Gel electrophoresis to show the validation of hTERT primer pairs using the following positive control samples: endometrial cancer 14, Hues7 ESCs and full thickness endometrium (proliferative phase)

It can be seen from the gel above that *TERT 1* gave rise to multiple product bands within endometrial cancer 14 tissue. *TERT 1* and *TERT 2* both failed to detect expression within the Hues7 ESCs. *TERT3* showed a consistent single band within all three sample types and therefore it was concluded that this was the best primer pair to use. *TERT 3* had also been used successfully by Dean Hallam, in our partner lab within Newcastle, who had optimised this primer on corneal tissue at an annealing temperature of 55°C. This annealing temperature was therefore adopted for the endometrial samples within this study, but quantifiable results could not be achieved due to low expression levels within these samples. Although an attempt to optimise the *TERT 3* primer set was made, this failed to provide quantifiable qPCR results. A literature search was performed and a fourth primer set, *TERT 4*, was discovered which had been used by Kim CM *et al* for qPCR on endometrial tissue taken from normal healthy women, and women with endometriosis (table 13) (Kim *et al*, 2007). As this primer set had proven successful on the same tissue used in our study, it was decided to run this under the same conditions on serial dilutions of a HeLa cell positive control, in order to construct a standard curve (figure 4.5).

Table 13 *hTERT* primer pair sequences (Pair 4)

Gene	Sequence	Product Size	Reference
TERT 4	F: 5'TGACACCTCACCTCACCCAC3' R: 5'CACTGTCTTCCGCAAGTTCAC3'	95	Kim CM, 2007
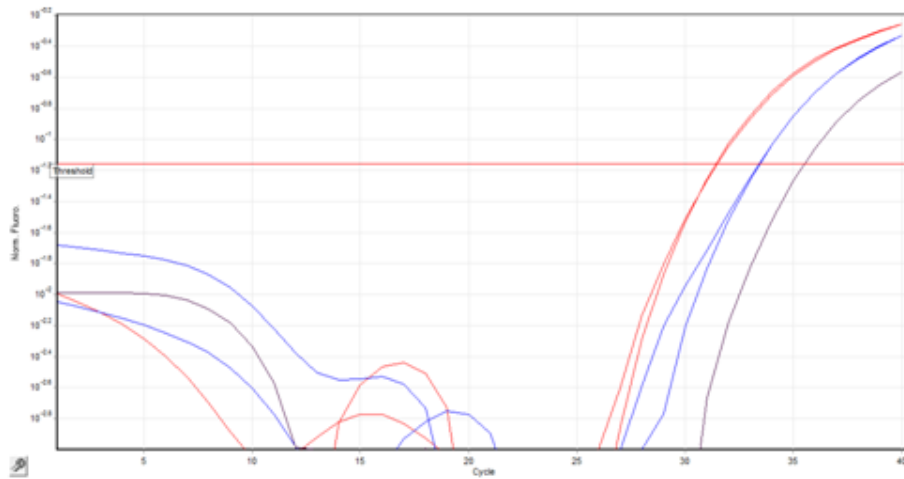
Figure 4.6 Standard curve for *hTERT*: 1x *hTERT* (red), 1/5x *hTERT* (blue), 1/25x *hTERT* (purple). Positive control template= HeLa cells. Primer efficiency= 1.21

Following this, the primer set was run on the sorted samples. Unfortunately quantifiable results again could not be achieved via qPCR when using this primer set. Instead, qualitative analysis was performed by running the PCR products on an agarose gel. The gel showed the expression of *hTERT* in both the SSEA-1$^+$ and SSEA-1$^-$ fractions. Comparing the intensity of the *hTERT* bands to *YWHAZ*, there appeared to be equal levels of *hTERT* in both cell populations in the majority of samples (samples 1, 4, 5 and 6). Samples 2 and 3 both seemed to express *hTERT* within the SSEA-1$^-$ cells only, whereas sample 7 looked to have more *hTERT* within the SSEA-1$^+$ population (see figure 4.7 below).
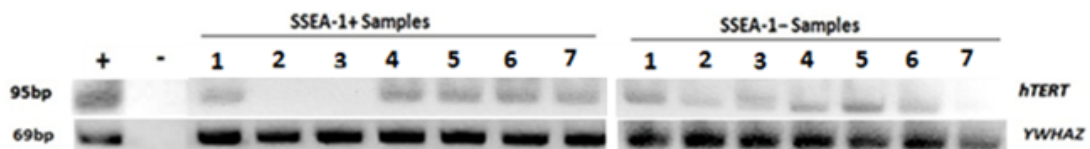
Figure 4.7 Gel electrophoresis: qualitative analysis of *hTERT* mRNA expression within normal MACS sorted SSEA-1 epithelial cells, sorted from 7 normal endometrial samples (labelled 1-7). *YWHAZ* was used as a reference. += positive control (endometrial cancer); -= negative control (NTC)

hTERT was ultimately excluded from any further qPCR experiments on additional samples, all of which had relatively low quantities of RNA.

It has been reported within the literature that techniques such as microdissection and immunomagnetic bead separation, used to enrich for a target cell population, make the detection of *hTERT* mRNA difficult (Lehner M, 2002). Methods of detection of *hTERT* transcripts within these samples are still not well established. The *hTERT* transcript has at least six alternative splice variants, including four insertions and two deletions (Lehner *et al*, 2002). The four insertion variants and the β-deletion variant all result in the premature termination of the translation of *hTERT* (Lehner *et al*, 2002). On the other hand, the α-deletion variant is a powerful inhibitor of telomerase expression and activity (Lehner *et al*, 2002). As telomerase protein is observed by IHC and telomerase activity is detected within the endometrial epithelium and SSEA-1 sorted cells, it seems that it was purely the low concentrations of *hTERT* mRNA rather than alternative splicing which made qPCR on these samples difficult and imprecise (Hapangama *et al*, 2008; Hapangama *et al*, 2009; Tanaka *et al*, 1998; Hapangama *et al*, unpublished results). It is also possible that in some samples, the low levels of *hTERT* may have been due to mRNA degradation. For accurate quantification of samples containing lower concentrations, the use of digital PCR may be considered, or the mRNA could be amplified prior to reverse transcriptase (Bustin *et al*, 2009).

3.2.2 leucine-rich repeat containing G protein-coupled receptor 5 (LGR5)

The human endometrium resembles the gastrointestinal system with its rapid turnover time, highly regenerative capacity and glandular components. It was for these reasons that *LGR5* was initially included in the preliminary investigations of this study, to establish whether it also expressed in endometrial epithelial cells. The *LGR5* qPCR primer sequences which had been selected from a previous publication were already

found available within the lab when starting this project. The sequence has been indicated in the following table (table 14).

Table 14 *LGR5* primer pair sequences

Gene	Sequence	Product Size	Reference
LGR5	F: 5'CTTCCAACCTCAGCGTCTTC3' R: 5'TTTCCCGCAAGACGTAACTC3'	118	Walker *et al*, 2011

This primer was initially tested on the human colon adenocarcinoma cell line HT29, as a positive control, and the following standard curve was generated (figures 4.8 and 4.9).
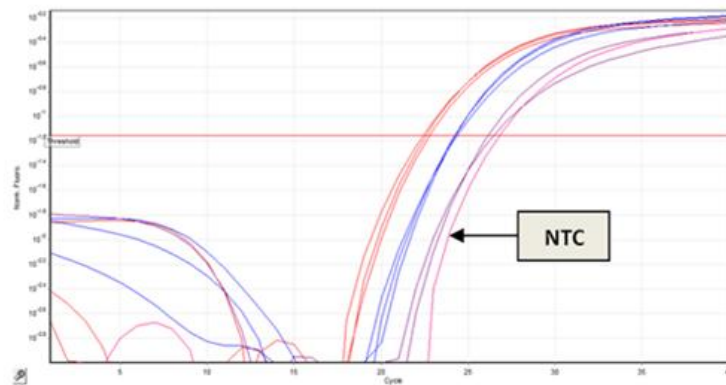
Figure 4.8 Standard curve for *LGR5*: 1x *LGR5* (red), 1/5x *LGR5* (blue), 1/25x *LGR5* (purple), NTC (pink). Positive control template= HT29 cells. Primer efficiency= 1.39.
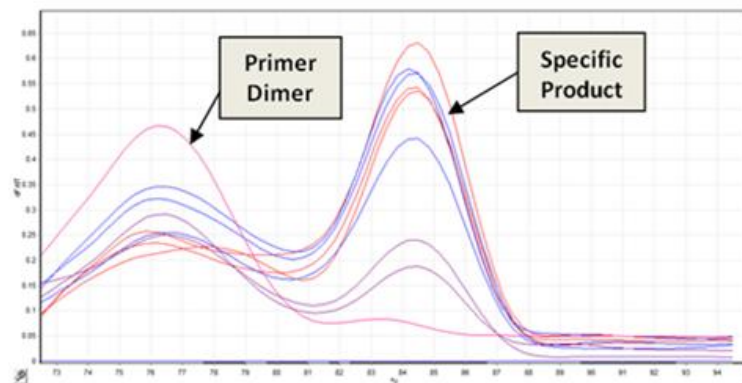
Figure 4.9 Melt curves for *LGR5*: 1x *LGR5* (red), 1/5x *LGR5* (blue), 1/25x *LGR5* (purple), NTC (pink). Positive control template= HT29 cells. Large primer dimer exposed when no template present

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It is evident from the graphs above that these primers gave rise to a large amount of unspecific primer-dimer product. When testing this primer set on the full thickness endometrium, very low levels of specific product were detected. Increasing the annealing temperature from 55°C to 60°C did not help to improve the specificity or efficiency of these primers (figure 5.0).
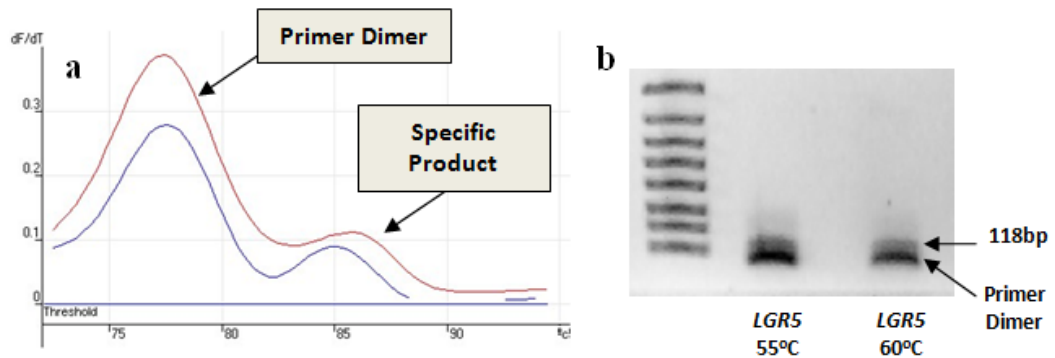
Figure 5.0 a) Melt curves for LGR5: Red line represents LGR5 at 55°C annealing temperature. Blue line represents LGR5 at 60°C annealing temperature. **b)** Agarose gel: Increasing annealing temperature has not affect on the amount of non-specific primer dimer product.

When proceeding to test the primer set on sorted stomal and epithelial endometrial cells, it became evident that *LGR5* was only expressed by the stromal cell population and not by endometrial epithelial cells (figure 5.1). As this study focuses on endometrial epithelial cells alone, *LGR5* was excluded from the study and its expression was not investigated within the SSEA-1 sorted epithelial cells.
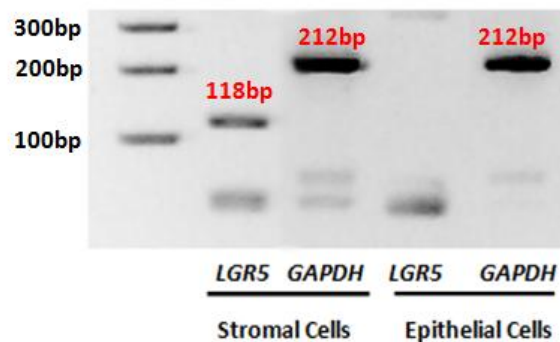
Figure 5.1 Gel electrophoresis: Comparison of *LGR5* mRNA expression between the stromal and epithelial cell populations of the endometrium

3.2.3 Oestrogen receptor beta (ERβ)

The action of oestrogen is modulated by two subtypes of oestrogen receptor, known as oestrogen receptor alpha (*ERα*) and oestrogen receptor beta (*ERβ*). Therefore it was initially planned that the expression of both of these receptors would be investigated within the SSEA-1 sorted endometrial epithelial cells. An *ERβ* primer set suitable for qPCR was selected from within the literature and has been identified within the table below (table 15). The authors of this paper are established experts in the study of *ERβ*.

Table 15 *ERβ1* primer pair sequences

Gene	Sequence	Product Size	Reference
ERβ1	F: 5'CCTGGCTAACCTCCTGATGCT3' R: 5' CCACATTTTTGCACTTCATGTTG 3'	92	Critchley *et al*, 2009

Critchley *et al* and others have reported that the level of *ERβ* mRNA is low within the endometrium (Critchley *et al*, 2009). Brandenberger *et al* established that *ERα* is 15 times more abundant that *ERβ* within normal endometrial stromal cells (Brandenberger *et al*, 1999). *ERβ* mRNA is expressed at significantly higher levels within the late secretory phase compared to any other time in the cycle. Studies by Critchley *et al* however have shown that the mRNA and protein levels for *ERβ* within the endometrium do not necessarily correlate. They found significant amounts of this nuclear protein within the tissue but low levels of mRNA, which was found to be in keeping with our findings (Critchley *et al*, 2009).

When testing the primers mentioned above on serial dilutions of a full thickness endometrial sample taken from the secretory phase, the following standard curve was generated (figures 5.2 and 5.3).
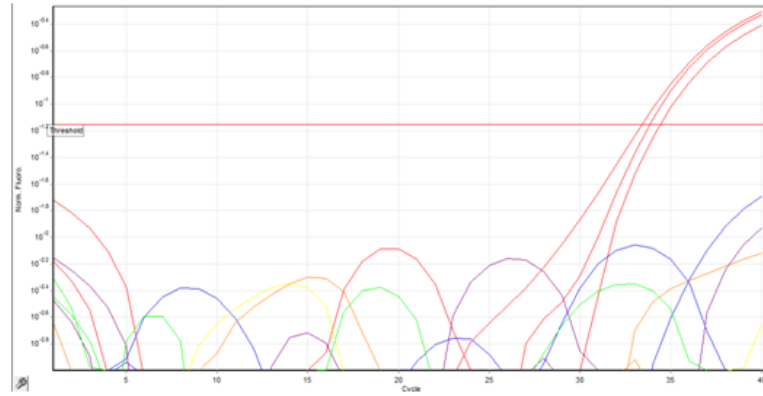
Figure 5.2 Standard curve for *ERβ*: 1x *ERβ*. Positive control template= secretory phase endometrium. *ERβ* mRNA was only detectable within the most concentrated sample
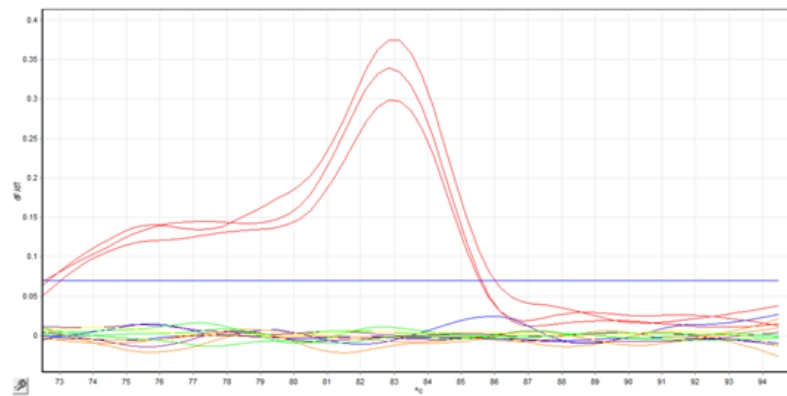
Figure 5.3 Melt curves for *ERβ*: 1x *ERβ* (red). Positive control template= secretory phase endometrium. *ERβ* mRNA was only detectable within the most concentrated sample

qPCR on the secretory phase endometrium (positive control) proved that *ERβ* mRNA is present at a very low level within the endometrium. For this reason, *ERβ* qPCR was not pursued on the sorted samples as concentrations would be significantly lower than the whole tissue samples. In contrast to the low mRNA levels, the following IHC images of the normal endometrium show that the *ERβ* protein is present in abundance (figure 5.4). This is consistent with the findings of previous studies (Critchley *et al*, 2009).
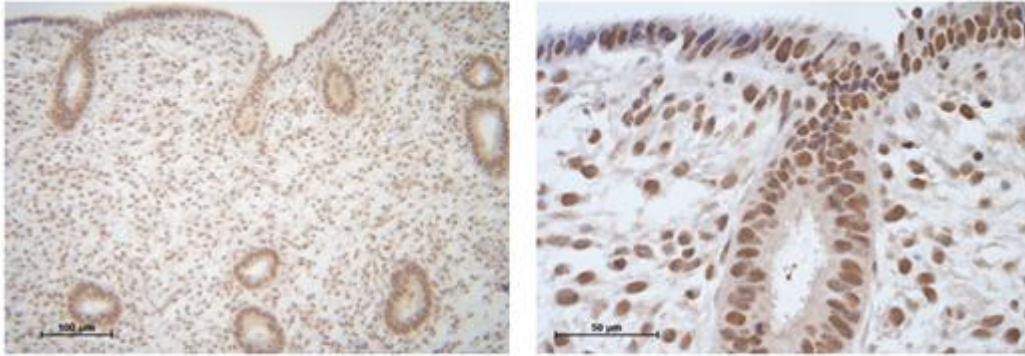
Figure 5.4 IHC staining of normal full thickness endometrium. Positive brown staining for *ERβ* found within the epithelial and stromal cellular compartments. Images taken at x16 (left) and x60 (right) magnifications (provided by Jo Drury, Liverpool Women's Hospital)

Chapter 4

Results

Chapter 4: Results

4.1 Patient Demographics

This study took place over a 12 month period, from the beginning of September 2011 until the end of August 2012. Over the entire duration of the study, samples collected from a total of 23 participants who were recruited for the study (reference 09/H1005/55 and 04/Q1505/112) were analysed. This included 14 normal fertile women who served as controls and 9 women with endometriosis. Of these, 2 control patients were excluded from the data analysis due to the exceedingly low and degraded RNA yields making them not suitable for analysis.

Patient Demographics:

Note that the 2 patients who were excluded from the data analyses have not been included in the table below.

Table 16 Demographic data for all patients included in the study

	Control Group (n=12)	Endometriosis Group (n=8)
Sample Type (no. patients)	**Pipelle:** 7 **Full thickness:** 5	**Pipelle:** 5 **Full thickness:** 3
Age (years)	**Median:** 40.5 **Range:** 30-49	**Median:** 39 **Range:** 27-48
Weight (kg)	**Median:** 76 **Range:** 56-111	**Median:** 70 **Range:** 52-103
Height (m)	**Median:** 1.605 **Range:** 1.51-1.84	**Median:** 1.635 **Range:** 1.55-1.71
BMI	**Median:** 29.55 **Range:** 24.5-39.8	**Median:** 25.25 **Range:** 20.5-37.8
Parity	**Median:** 2 **Range:** 0-4	**Median:** 1 **Range:** 0-4
Gravidity	**Median:** 2 **Range:** 0-4	**Median:** 1 **Range:** 0-4
Cycle Stage (no. patients)	**Proliferative:** 4 **Secretory:** 5 (Missing data)	**Mid-cycle:** 3 **Secretory:** 5
Endometriosis Stage (no. patients)	Not Applicable	**Stage 1:** 2 **Stage 3:** 0 **Stage 2:** 1 **Stage 4:** 4 (Missing data)

A couple of patient demographic details were missing from this data set and are currently being located (missing data indicated in the table above). In order to assess whether there were any statistical differences in patient demographics at baseline between the control group (with normal endometrial tissue) and patients with endometriosis, statistical analysis has been conducted. This is important as if baseline differences between patient groups are not accounted and adjusted for, direct comparison between the two groups may be invalid. Due to the low number of patients within each group, non-parametric statistical tests were used to analyse our data throughout this study. Using the MWU test for nonparametric independent groups, it was confirmed that there were no statistical differences ($p > 0.05$) between any of the demographic variables documented in the table above. Direct comparison between the control and pathological groups was therefore valid and no adjustments were needed at baseline.

Table 17: P-values for demographic variables between groups; MWU test

Demographic Variable	P-value
Age	0.9692
Weight	0.1888
Height	0.7669
BMI	0.0698
Parity	0.1752
Gravidity	0.113

From one patient who was recruited into the study, multiple samples could be taken for use in several lab experiments and techniques. From the 21 patients mentioned previously, 14 samples were MACS sorted (7 normal endometrium, 7 endometriosis), 3 were FACS sorted (all normal endometrium), 6 were grown into organoids in 3D culture (3 normal endometrium, 3 endometriosis) and 1 was used for IF staining (normal endometrium).

4.2 mRNA Data: SSEA-1$^+$ versus SSEA-1$^-$ Endometrial Epithelial Cell Population

qPCR was used to compare the mRNA levels of the stem cell markers *OCT4, NANOG, SOX2, CD133* and *PODXL* within the SSEA-1 sorted epithelial cells taken from the normal endometrium. The expression of markers of differentiation, *PR* and *ERα*, and the epithelial cell marker, *CD9*, were also analysed within these samples. *FUT4* was included in the gene expression profile of these samples as its function is said to include the fucosylation of the carbohydrate structure SSEA-1 (Ponnampalam *et al*, 2008) hence may be a surrogate marker for SSEA-1. As SSEA-1 is a carbohydrate, it could not be looked at directly using qPCR.

4.2.1 MACS Sorted Normal Endometrium (n=7)

Seven samples were MACS sorted to generate an SSEA-1$^+$ and an SSEA-1$^-$ epithelial cell population for each sample. The presence of all genes was confirmed within both SSEA-1 MACS sorted fractions for all samples, excluding *SOX2* (figure 5.5). *SOX2* was found to be absent across all samples. This was consistent with IHC analysis of full thickness endometrial tissue sections performed at Liverpool Women's Hospital laboratory, which did not find the *SOX2* protein anywhere within the normal full thickness endometrium (figure 5.6). The absence of *SOX2* within the endometrium was also supported by the negative qPCR results on non-sorted normal full thickness endometrium.
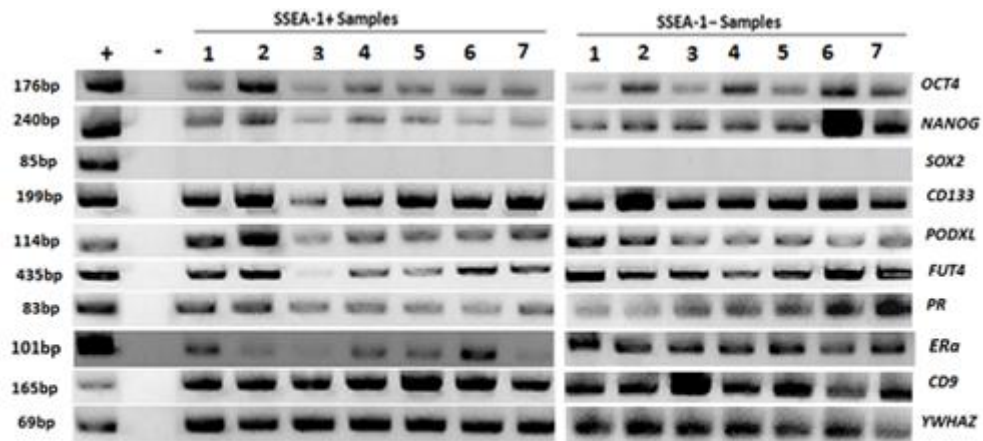
Figure 5.5 Agarose gel to confirm the presence of all genes of the correct product size, except *SOX2*, across all 7 SSEA-1 MACS sorted normal endometrial samples (labelled 1-7)
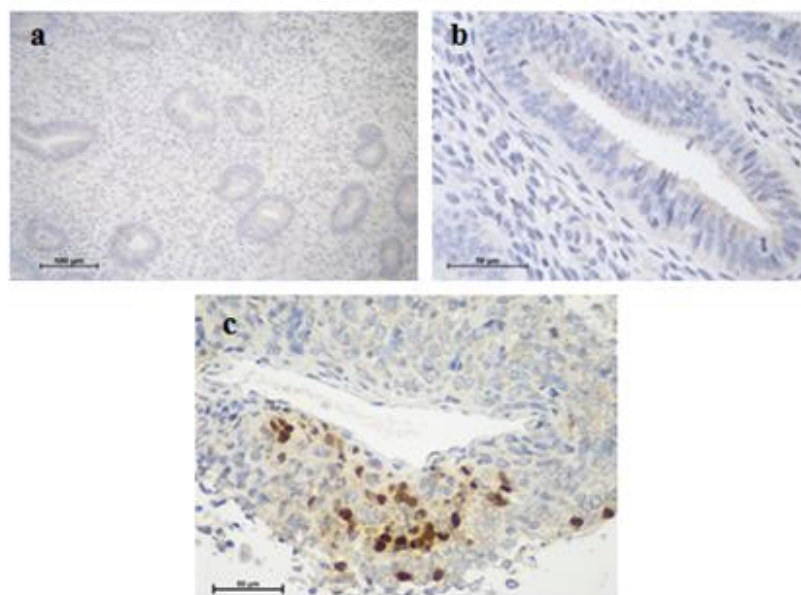
Figure 5.6 *SOX2* IHC staining: negative staining throughout the normal full thickness endometrium a) x16 magnification b) x60 magnification c) *SOX2* endometrial cancer positive control (x40 magnification) (provided by Jo Drury and Lisa Heathcote, Liverpool Women's Hospital)

When comparing quantitative data for the genes expressed above in the normal endometrium, no significant differences were seen between the expression levels of the stem cell markers *OCT4, NANOG, PODXL* and *CD133* in the SSEA-1^{+} and the SSEA-1^{-} epithelial cell fractions. Of these stem cell markers, *PODXL* mRNA was most highly expressed by the sorted samples with a median relative expression level of 0.26 and 0.4 (WSR test; p=0.8125) in the SSEA-1^{+} and SSEA-1^{-} fractions respectively.

OCT4 mRNA was also highly expressed by both fractions with median relative expression levels of 0.2 within both populations (WSR test; p=0.2188). Comparably, *CD133* and *NANOG* were both expressed to a lower degree by both fractions with median relative expression levels of 0.01-0.02 (WSR test; p=0.8125) and 1.0×10^{-4} (WSR test; p=0.2188) respectively (see results in figure 5.7).

A) Expression of *OCT4* relative to *YWHAZ*

p-value= 0.2188		
	SSEA-1⁺	SSEA-1⁻
Median	0.21	0.23
IQR	0.15-0.22	0.13-0.36
Mean	0.19	0.24
SD	0.053	0.12
95% CI	0.14-0.24	0.13-0.38

B) Expression of *NANOG* relative to *YWHAZ*

p-value= 0.2188		
	SSEA-1⁺	SSEA-1⁻
Median	1.0×10^{-4}	1.0×10^{-4}
IQR	4.7×10^{-5}-1.1×10^{-4}	7.6×10^{-5}-1.9×10^{-4}
Mean	9.4×10^{-5}	1.3×10^{-4}
SD	5.9×10^{-5}	6.8×10^{-5}
95% CI	3.9×10^{-5}-1.5×10^{-4}	6.7×10^{-5}-1.9×10^{-4}

C) Expression of *PODXL* relative to *YWHAZ*

p-value= 0.8125		
	SSEA-1⁺	SSEA-1⁻
Median	0.26	0.40
IQR	0.19-0.64	0.22-0.67
Mean	0.52	0.42
SD	0.54	0.22
95% CI	0.018-1.017	0.21-0.62

D) Expression of *CD133* relative to *YWHAZ*

p-value= 0.8125		
	SSEA-1⁺	SSEA-1⁻
Median	0.015	0.013
IQR	0.009-0.026	0.01-0.037
Mean	0.019	0.02
SD	0.016	0.014
95% CI	0.0045-0.033	0.0065-0.033

Figure 5.7 qPCR data of *a) OCT4 b) NANOG c) PODXL d) CD133* expression relative to the reference gene YWHAZ in SSEA-1 MACS sorted normal epithelial cells grown in 2D culture. Height of bars represents median values and error bars represent interquartile ranges (IQRs) as summary measures for non- parametric data

For markers of differentiation, the expression of *ERα* and *PR* mRNA were both significantly greater within the SSEA-1⁻ epithelial fraction (WSR test; p=0.0156). For *ERα* and *PR*, expression was approximately 3-fold and 5-fold greater respectively within the SSEA-1⁻ population (see results in figure 5.8).

p-value= 0.0156		
	SSEA-1⁺	SSEA-1⁻
Median	0.0098	0.029
IQR	0.0077-0.024	0.016-0.034
Mean	0.013	0.025
SD	0.0081	0.012
95% CI	0.0056-0.020	0.014-0.036

p-value= 0.0156		
	SSEA-1⁺	SSEA-1⁻
Median	0.012	0.046
IQR	0.0063-0.019	0.020-0.053
Mean	0.014	0.059
SD	0.011	0.068
95% CI	0.0038-0.024	-0.0038-0.12
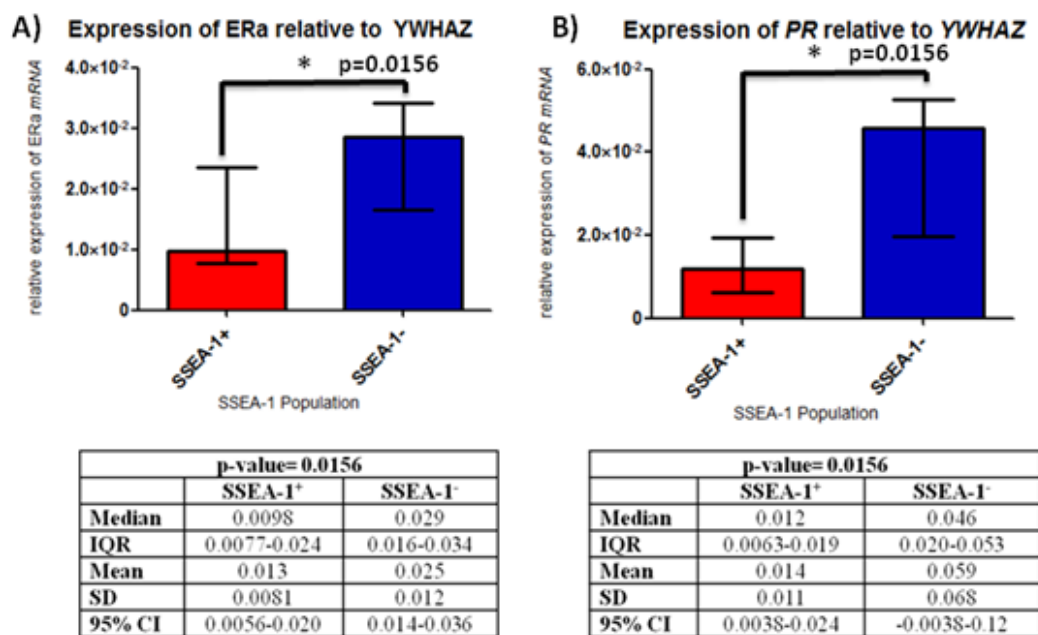
Figure 5.8 qPCR data of *A) ERα B) PR* expression relative to the reference gene YWHAZ in SSEA-1 MACS sorted normal epithelial cells grown in 2D culture. Height of bars represents median values and error bars represent interquartile ranges (IQR) as summary measures for non-parametric data

For other markers, *CD9* and *FUT4*, no significant differences in expression levels were noted between the SSEA-1 sorted populations (WSR test; p=0.9375) (see results in figure 5.9).

| | **A)** Expression of *CD9* relative to *YWHAZ* | | | **B)** Expression of *FUT4* relative to *YWHAZ* | |

	p-value= 0.9375	
	SSEA-1⁺	**SSEA-1⁻**
Median	12.56	15.25
IQR	9.49-31.68	13.77-21.63
Mean	17.60	17.49
SD	10.37	4.35
95% CI	8.019-27.19	13.47-21.51

	p-value= 0.9375	
	SSEA-1⁺	**SSEA-1⁻**
Median	0.074	0.067
IQR	0.055-0.10	0.060-0.11
Mean	0.080	0.079
SD	0.029	0.031
95% CI	0.053-0.11	0.050-0.11
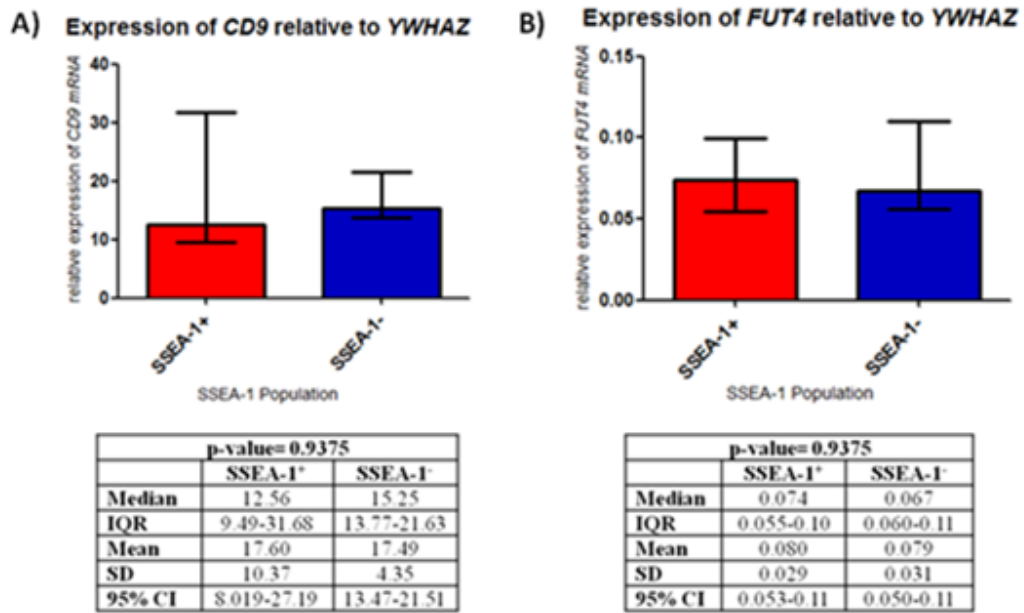
Figure 5.9 qPCR data of *A) CD9 B) FUT4* expression relative to the reference gene YWHAZ in SSEA-1 MACS sorted normal epithelial cells grown in 2D culture. Height of bars represents median values and error bars represent interquartile ranges (IQRs) as summary measures for non-parametric data

4.2.2 MACS Sorted Endometriosis Samples (n=7)

qPCR on seven MACS sorted samples taken from women with endometriosis showed similar mRNA results between the SSEA-1⁺ and SSEA-1⁻ epithelial cell populations as seen in the normal endometrium. All samples expressed all genes except for *SOX2*, which again was absent within the pathological endometrium. For stem markers *OCT4, NANOG, CD133* and *PODXL*, again there were no significant differences in expression between the SSEA-1 MACS sorted populations. Median relative expression of *PODXL* was the highest, with levels of 0.59 and 0.73 within the SSEA-1⁺ and SSEA-1⁻ populations respectively (WSR test; p=0.4688). *OCT4* expression was also relatively high at median levels of 0.42 and 0.75 within the SSEA-1⁺ and SSEA-1⁻ populations respectively (WSR test; p=2969). For *CD133* and *NANOG* relative expression was again seen at lower levels (see results in figure 6.0).

A) Expression of *OCT4* relative to *YWHAZ*
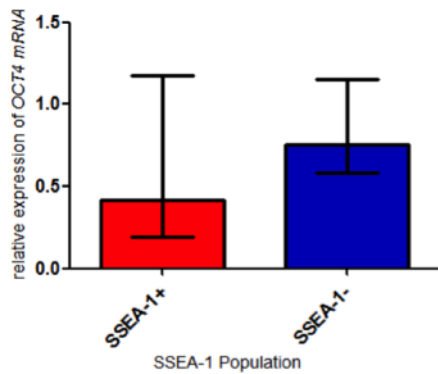
p-value= 0.2969		
	SSEA-1$^+$	SSEA-1$^-$
Median	0.42	0.75
IQR	0.20-1.17	0.18-1.16
Mean	0.67	0.93
SD	0.48	0.67
95% CI	0.22-1.11	0.31-1.55

B) Expression of *NANOG* relative to *YWHAZ*
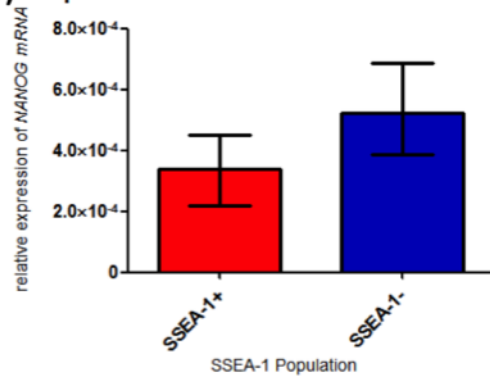
p-value= 0.1094		
	SSEA-1$^+$	SSEA-1$^-$
Median	3.4×10^{-4}	5.2×10^{-4}
IQR	2.2×10^{-4}-4.5×10^{-4}	3.9×10^{-4}-6.9×10^{-4}
Mean	3.2×10^{-4}	5.2×10^{-4}
SD	1.5×10^{-4}	1.9×10^{-4}
95% CI	1.8×10^{-4}-4.6×10^{-4}	3.5×10^{-4}-7.0×10^{-4}

C) Expression of *PODXL* relative to *YWHAZ*

p-value= 0.4688		
	SSEA-1$^+$	SSEA-1$^-$
Median	0.59	0.73
IQR	0.37-0.84	0.47-0.95
Mean	0.85	0.83
SD	0.89	0.46
95% CI	0.018-1.67	0.40-1.25

D) Expression of *CD133* relative to *YWHAZ*
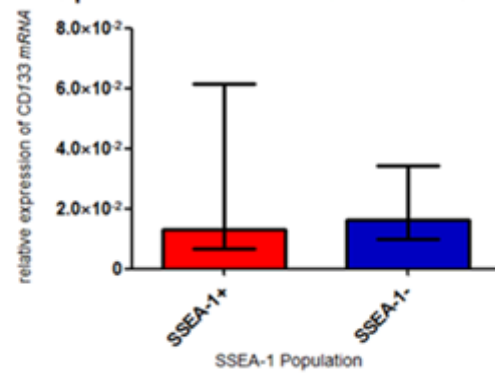
p-value= 0.8125		
	SSEA-1$^+$	SSEA-1$^-$
Median	0.013	0.016
IQR	0.0066-0.061	0.0099-0.034
Mean	0.029	0.023
SD	0.026	0.018
95% CI	0.0048-0.03	0.0057-0.040

Figure 6.0 qPCR data of *A) OCT4 B) NANOG C) PODXL D) CD133* expression relative to the reference gene *YWHAZ* in SSEA-1 MACS sorted endometriosis epithelial cells grown in 2D culture. Height of bars represents median values and error bars represent interquartile ranges (IQRs) as summary measures for non-parametric data

For markers of differentiation, *PR* showed similar results to those found in the normal endometrium. A statistically significant 2-fold increase (WSR test; p=0.0156) in *PR* mRNA expression was found within the SSEA-1$^-$ epithelial cell population when

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compared to the SSEA-1$^+$ population. Unlike the normal endometrium however, no significant difference in *ERα* expression was noted between the sorted populations although expression was still 1.7-fold higher in the SSEA-1$^-$ population (see figure 6.1).

A) Expression of ERa relative to YWHAZ

B) Expression of *PR* relative to *YWHAZ*

	p-value= 0.0781	
	SSEA-1$^+$	SSEA-1$^-$
Median	0.0087	0.015
IQR	0.0073-0.014	0.0057-0.017
Mean	0.010	0.014
SD	0.0048	0.0067
95% CI	0.0059-0.015	0.0076-0.02

	p-value= 0.0156	
	SSEA-1$^+$	SSEA-1$^-$
Median	0.0090	0.020
IQR	0.0063-0.010	0.015-0.069
Mean	0.0095	0.036
SD	0.0049	0.031
95% CI	0.0050-0.014	0.0078-0.064
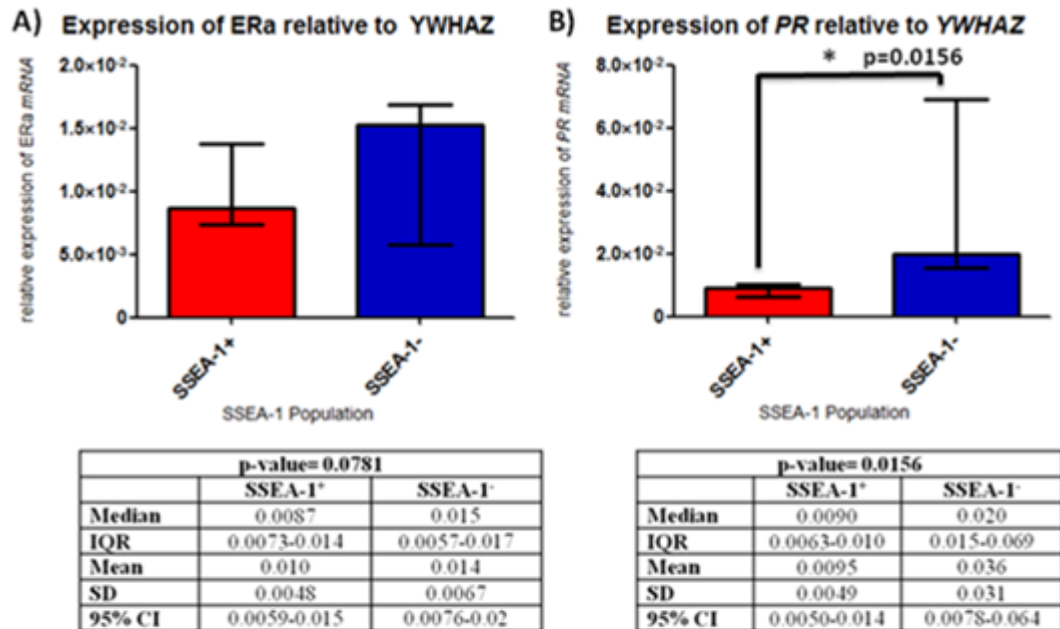
Figure 6.1 qPCR data of **A) ERα B) PR** expression relative to the reference gene YWHAZ in SSEA-1 MACS sorted endometriosis epithelial cells grown in 2D culture. Height of bars represents median values and error bars represent interquartile ranges (IQRs) as summary measures for non-parametric data

Similar to the results found within the normal fertile endometrium, *CD9* and *FUT4*, were both expressed at equal levels within endometrial epithelial cells sorted according to the expression of SSEA-1 taken from women with endometriosis. *FUT4* showed a median expression level of 0.18 in both sorted fractions (WSR test; p=0.4688) (see figure 6.2).

A) Expression of *CD9* relative to *YWHAZ*

B) Expression of *FUT4* relative to *YWHAZ*

	p-value= 0.9375	
	SSEA-1⁺	SSEA-1⁻
Median	19.22	17.73
IQR	10.48-25.73	11.15-29.43
Mean	19.98	21.60
SD	10.67	13.07
95% CI	10.11-29.84	9.511-33.68

	p-value= 0.4688	
	SSEA-1⁺	SSEA-1⁻
Median	0.18	0.18
IQR	0.086-0.38	0.10-0.25
Mean	0.21	0.17
SD	0.13	0.069
95% CI	0.094-0.33	0.11-0.24

Figure 6.2 qPCR data of *A) CD9 B) FUT4* expression relative to the reference gene YWHAZ in SSEA-1 MACS sorted endometriosis epithelial cells grown in 2D culture. Height of bars represents median values and error bars represent interquartile ranges (IQRs) as summary measures for non-parametric data

4.2.3 FACS Sorted Normal Endometrium (n=3)

As MACS sorting only achieves up to 70-75% enrichment of SSEA-1 in the SSEA-1⁺ fraction, the accuracy of the qPCR results may have been affected by contaminating SSEA-1+ cells and stromal cells which may have remained within the SSEA-1 depleted cell fraction. This may have had a significant effect on the results, especially if the contaminating cells were stromal stem cells or SSEA-1⁺ epithelial cells. It was therefore important to verify whether any true differences in gene expression seen between pure SSEA-1⁺ and SSEA-1⁻ cell populations had been masked/exaggerated in the previous results by repeating the qPCR experiments on FACS sorted normal endometrial samples. FACS sorting for both SSEA-1+ and the epithelial marker *CD9+*, is over 90% efficient in picking up cells that express both SSEA-1 and CD9 therefore are more likely to be epithelial cells that express SSEA-1. We believe, therefore qPCR results achieved from these samples are a more accurate

representation. Unfortunately only a limited number of normal endometrial samples could be sorted using FACS throughout the duration of this project due to technical difficulties (the inaccessibility of the FACS machine) and the requirement for a high cell yield. A representative FACS profile of the sorted epithelial cells is shown in figure 6.3 below.
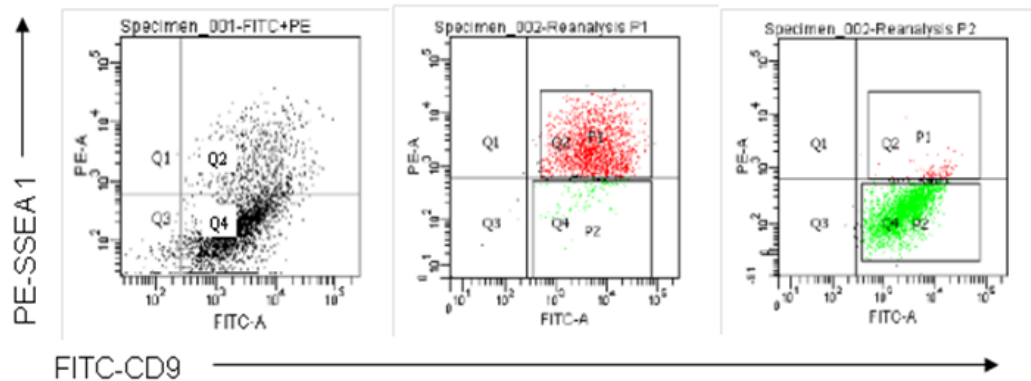
Figure 6.3 FACS analysis of epithelial cells dual labelled for SSEA-1 and CD9
(A) Epithelial cells grown in monolayer were labelled with PE-SSEA-1 and FITC-CD9 and sorted by FACS into a SSEA-1+/CD9+ fraction and a SSEA-1-/CD9+ fraction. Representative dot blot of the analyses (left panel), re-analysis of the SSEA-1+/CD9+ sorted fraction (middle panel) and re-analysis of the SSEA-1-/CD9+ (right panel) (provided by Anthony Valentijn, Liverpool Women's Hospital)

Gene expression analysis on three FACS sorted normal endometrial samples confirmed that there were no differences in the mRNA levels of *OCT4, NANOG, PODXL, CD133, CD9* and *FUT4* between the SSEA-1$^+$/CD9+ and SSEA-1$^-$/CD9- cell populations. Although statistical significance was not achieved, potentially due to the low number of samples/cells, unlike the results obtained with MACS, a 2-fold greater level of *NANOG* mRNA expression was seen within the FACS-sorted SSEA-1$^+$/CD9+ population compared to the SSEA-1$^-$/CD9- population. Contrary to the previous results, data also showed that there were no differences in the expression levels of the differentiation markers *ERα* and *PR*, although there was still a 3.7 and 2-fold greater

expression of these genes respectively within the SSEA-1⁻ fraction (see figures 6.4 and 6.4 continued).

A) Expression of *OCT4* relative to *YWHAZ*

	p-value= 0.2500	
	CD9⁺SSEA-1⁺	CD9⁺SSEA-1⁻
Median	0.16	0.23
IQR	0.13-0.22	0.22-0.25
Mean	0.17	0.23
SD	0.045	0.016
95% CI	0.054-0.28	0.19-0.27

B) Expression of *NANOG* relative to *YWHAZ*

	p-value= 0.5	
	CD9⁺SSEA-1⁺	CD9⁺SSEA-1⁻
Median	3.9×10^{-4}	2.0×10^{-4}
IQR	8.0×10^{-5}-9.2×10^{-4}	1.6×10^{-4}-5.2×10^{-4}
Mean	4.6×10^{-4}	2.9×10^{-4}
SD	4.3×10^{-4}	2.0×10^{-4}
95% CI	-6.0×10^{-4}-1.5×10^{-3}	-2.0×10^{-4}-7.8×10^{-4}

C) Expression of *PODXL* relative to *YWHAZ*

	p-value= 0.5000	
	CD9⁺SSEA-1⁺	CD9⁺SSEA-1⁻
Median	2.33	3.92
IQR	1.26-2.62	0.6-5.43
Mean	2.07	3.32
SD	0.72	2.47
95% CI	0.29-3.85	-2.82-9.46

D) Expression of *CD133* relative to *YWHAZ*

	p-value= 1.0000	
	CD9⁺SSEA-1⁺	CD9⁺SSEA-1⁻
Median	0.0077	0.0065
IQR	0.00028-0.073	0.00038-0.0092
Mean	0.027	0.0054
SD	0.04	0.0045
95% CI	-0.072-0.13	-0.0059-0.017

Figure 6.4 qPCR data of *A) OCT4 B) NANOG C) PODXL D) CD133* expression relative to the reference gene YWHAZ in SSEA-1 FACS sorted normal epithelial cells grown in 2D culture. Height of bars represents median values and error bars represent interquartile ranges (IQRs) as summary measures for non-parametric data

E) Expression of *ERa* relative to *YWHAZ*
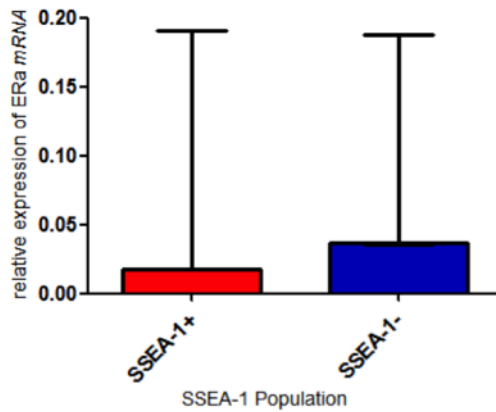
	p-value= 0.5000	
	CD9⁺SSEA-1⁺	CD9⁺SSEA-1⁻
Median	0.018	0.036
IQR	0.018-0.19	0.035-0.19
Mean	0.076	0.087
SD	0.1	0.088
95% CI	-0.17-0.32	-0.13-0.3

F) Expression of *PR* relative to *YWHAZ*
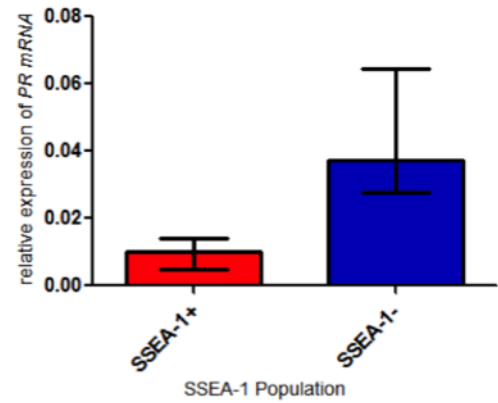
	p-value= 0.2500	
	CD9⁺SSEA-1⁺	CD9⁺SSEA-1⁻
Median	0.01	0.037
IQR	0.0048-0.014	0.028-0.064
Mean	0.01	0.043
SD	0.0047	0.019
95% CI	-0.002-0.021	-0.0044-0.09

G) Expression of *CD9* relative to *YWHAZ*
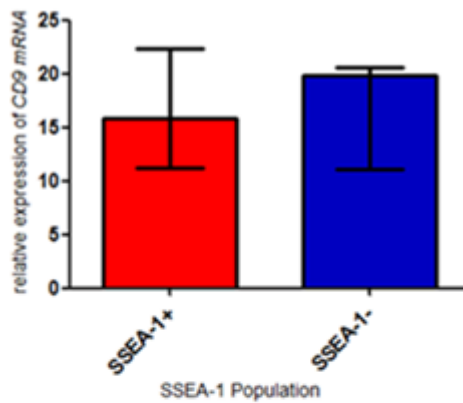
	p-value= 1.0000	
	CD9⁺SSEA-1⁺	CD9⁺SSEA-1⁻
Median	15.85	19.78
IQR	11.15-22.33	11.11-20.54
Mean	16.45	17.14
SD	5.61	5.24
95% CI	2.5-30.39	4.13-30.15

H) Expression of *FUT4* relative to *YWHAZ*

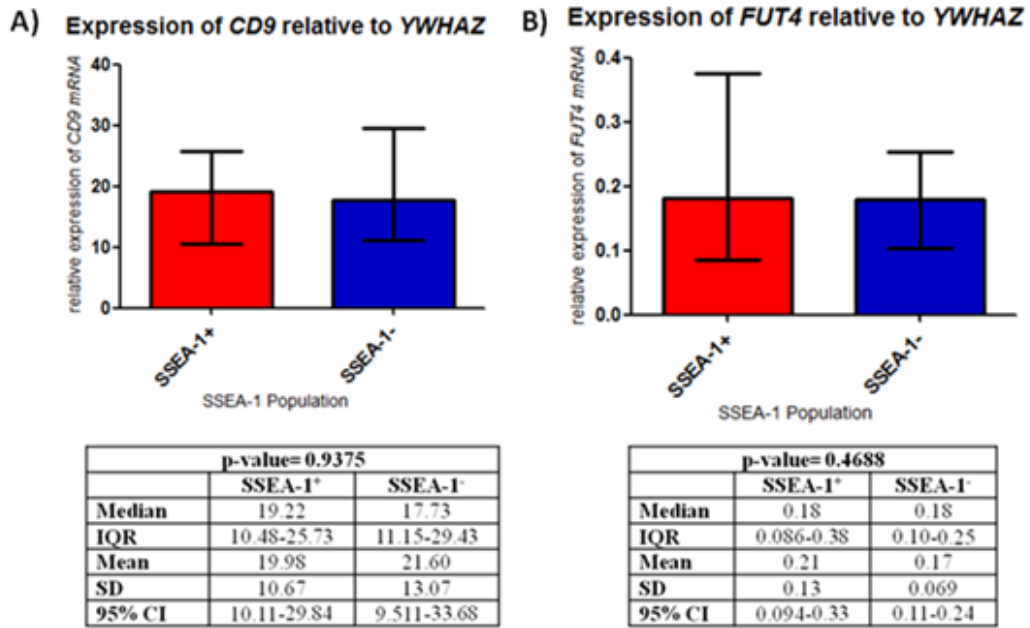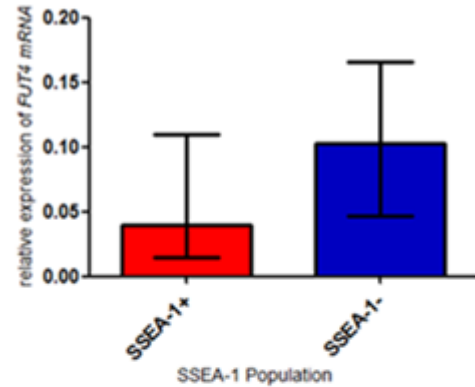|	p-value= 0.2500	
	CD9⁺SSEA-1⁺	CD9⁺SSEA-1⁻
Median	0.039	0.10
IQR	0.014-0.11	0.047-0.17
Mean	0.055	0.11
SD	0.05	0.06
95% CI	-0.069-0.18	-0.042-0.25

Figure 6.4 continued qPCR data of *E) ERa F) PR G) CD9 H) FUT4* expression relative to the reference gene *YWHAZ* in SSEA-1 FACS sorted normal epithelial cells grown in 2D culture. Height of bars represent median values and error bars represent interquartile ranges (IQRs) as summary measure for non-parametric data

4.3 mRNA Data: Comparison of Gene Expression within SSEA-1$^+$ Endometrial Epithelial Cells taken from Healthy Women and Women with Endometriosis

As it is postulated that the epithelial SPC resides within the SSEA-1$^+$ epithelial cell fraction, and that abnormal stem cells are implicated in the pathogenesis of endometriosis, we progressed to compare the SSEA-1$^+$ populations between normal and pathological samples.

Direct comparisons between the mRNA levels for the particular genes under investigation within this study indicated an increase in expression of the stem cell markers *OCT4*, *NANOG* and *PODXL* within the eutopic endometriosis samples compared to those taken from healthy, fertile patients. This reached significance for markers *OCT4* (WSR test; p=0.0469) and *NANOG* (WSR test; p=0.0313) with a 2 and 3.4-fold increase respectively in mRNA levels within the endometriosis samples. *PODXL* showed a 2.3 fold increase within the endometriosis samples, although not significant. *CD133* levels show no difference between the two groups (see figure 6.5).

A) Expression of *OCT4* relative to *YWHAZ*
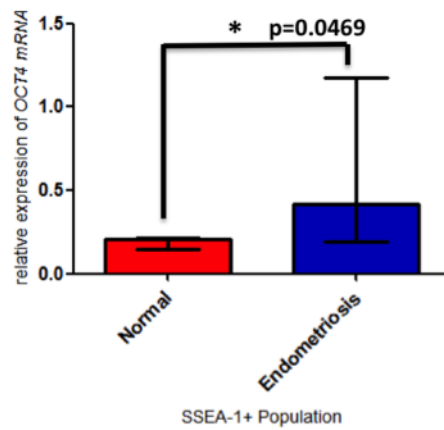
	p-value= 0.0469	
	Normal	Endometriosis
Median	0.21	0.42
IQR	0.15-0.22	0.20-1.17
Mean	0.19	0.67
SD	0.053	0.48
95% CI	0.14-0.24	0.22-1.11

B) Expression of *NANOG* relative to *YWHAZ*
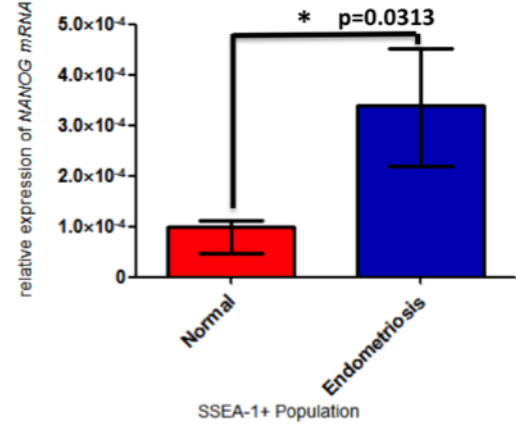
	p-value= 0.0313	
	Normal	Endometriosis
Median	1.0×10^{-4}	3.4×10^{-4}
IQR	4.7×10^{-5}-1.1×10^{-4}	2.2×10^{-4}-4.5×10^{-4}
Mean	9.4×10^{-5}	3.2×10^{-4}
SD	5.9×10^{-5}	1.5×10^{-4}
95% CI	3.9×10^{-5}-1.5×10^{-4}	1.8×10^{-4}-4.6×10^{-4}

C) Expression of *PODXL* relative to *YWHAZ*

	p-value= 0.5781	
	Normal	Endometriosis
Median	0.26	0.59
IQR	0.19-0.64	0.37-0.84
Mean	0.52	0.85
SD	0.54	0.89
95% CI	0.018-1.02	0.018-1.67

D) Expression of *CD133* relative to *YWHAZ*
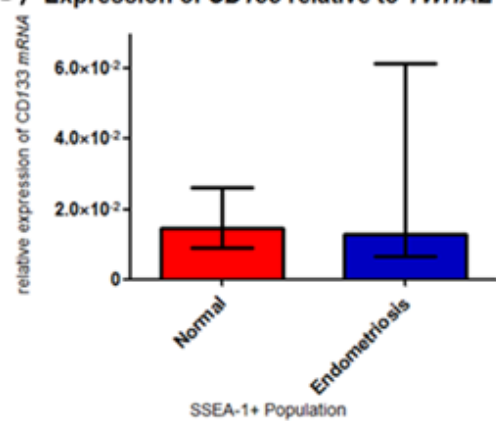
	p-value= 0.2969	
	Normal	Endometriosis
Median	0.015	0.013
IQR	0.0090-0.026	0.0066-0.061
Mean	0.019	0.029
SD	0.016	0.026
95% CI	0.0045-0.033	0.0048-0.053

Figure 6.5 qPCR data of **A)** *OCT4* **B)** *NANOG* **C)** *PODXL* **D)** *CD133* expression relative to the reference gene *YWHAZ* in SSEA-1⁺ MACS sorted normal and endometriosis epithelial cells grown in 2D culture. Height of bars represents median values and error bars represent interquartile ranges (IQRs) as summary measures for non-parametric data

Markers of differentiation showed no significant differences between the expression of *ERα* and *PR* mRNA within endometriosis or normal endometrial tissue (see figure 6.6).

Figure 6.6 panels:

A) Expression of ERa relative to YWHAZ (SSEA-1+ Population)

	p-value= 0.6875	
	Normal	**Endometriosis**
Median	0.0098	0.0087
IQR	0.0077-0.024	0.0073-0.014
Mean	0.013	0.010
SD	0.0081	0.0048
95% CI	0.0056-0.020	0.0059-0.015

B) Expression of PR relative to YWHAZ (SSEA-1+ Population)

	p-value= 0.5781	
	Normal	**Endometriosis**
Median	0.012	0.0090
IQR	0.0063-0.019	0.0063-0.010
Mean	0.014	0.0095
SD	0.011	0.0049
95% CI	0.0038-0.024	0.0050-0.014

Figure 6.6 qPCR data of **A) ERα B) PR** expression relative to the reference gene *YWHAZ* in SSEA-1⁺ MACS sorted normal and endometriosis epithelial cells grown in 2D culture. Height of bars represents median values and error bars represent interquartile ranges (IQRs) as summary measures for non-parametric data

A significant (WSR test; p=0.0469) 2.6-fold increase in *FUT4* expression was seen within endometriosis samples compared to the normal samples. *CD9* mRNA expression was only slightly increased in endometriosis samples (see figure 6.7).

A) Expression of *CD9* relative to *YWHAZ*

B) Expression of *FUT4* relative to *YWHAZ*

	p-value= 0.3750	
	Normal	**Endometriosis**
Median	12.56	19.22
IQR	9.49-31.68	10.48-25.73
Mean	17.60	19.98
SD	10.37	10.67
95% CI	8.02-27.19	10.11-29.84

	p-value= 0.0469	
	Normal	**Endometriosis**
Median	0.074	0.18
IQR	0.055-0.10	0.086-0.38
Mean	0.080	0.21
SD	0.029	0.13
95% CI	0.053-0.11	0.094-0.33

Figure 6.7 qPCR data of *A) CD9 B) FUT4* expression relative to the reference gene YWHAZ in SSEA-1+MACS sorted normal and endometriosis epithelial cells grown in 2D culture. Height of bars represents median values and error bars represent interquartile ranges (IQRs) as summary measures for non-parametric data

4.4 mRNA Data: Normal and endometriosis SSEA-1 sorted epithelial cells grown in 3D culture (organoids)

SSEA-1 sorted epithelial cells have been shown to form gland-like structures (which we called as spheroids/organoids) in 3D culture, and they have structural similarities to the endometrial glands seen *in vivo*. These organoids arise primarily from the MACS sorted SSEA-1$^+$ epithelial fraction, with significantly fewer and smaller organoids being formed by the SSEA-1$^-$ cell population. 2D culture has many limitations when studying endometrial cells, and the morphology of the cells seems to be very different to those seen *in vivo*. The Matrigel acts as an ECM which imitates the architecture of the normal human endometrium, and may help to preserve the stemness of any SPCs which may be contained within the cell population on the one hand; and also may allow differentiation of the cells to create an endometrium like environment hence provide a stem cell niche to contain a stem cell (Zhu *et al*, 2012).

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Organoids, made up of clusters of a small number of cells, gave rise to low RNA yields. This made the accurate study of genes that do not show high mRNA expression levels, such as stem cell markers, via qPCR very difficult. For this reason, organoids grown from SSEA-1 sorted cells were pooled from three patients to achieve a higher RNA concentration. Unfortunately, this meant that statistical analysis of this data was not possible. qPCR was performed on the gene panel previously mentioned on two SSEA-1 sorted samples, one pooled from three patients with a normal endometrium and the second pooled from three patients with endometriosis.

The qPCR results obtained from these samples were different from those obtained from the 2D-cultured samples. In 3D-culture, the stem cell markers *OCT4, NANOG, PODXL* and CD133, were seen to be raised within the SSEA-1$^+$ organoids when compared to the SSEA-1$^-$ organoids. The difference between the sorted populations was even more pronounced within women with endometriosis, with expression greatly increased for most of these genes within the SSEA-1$^+$ organoids (see figure 6.8).
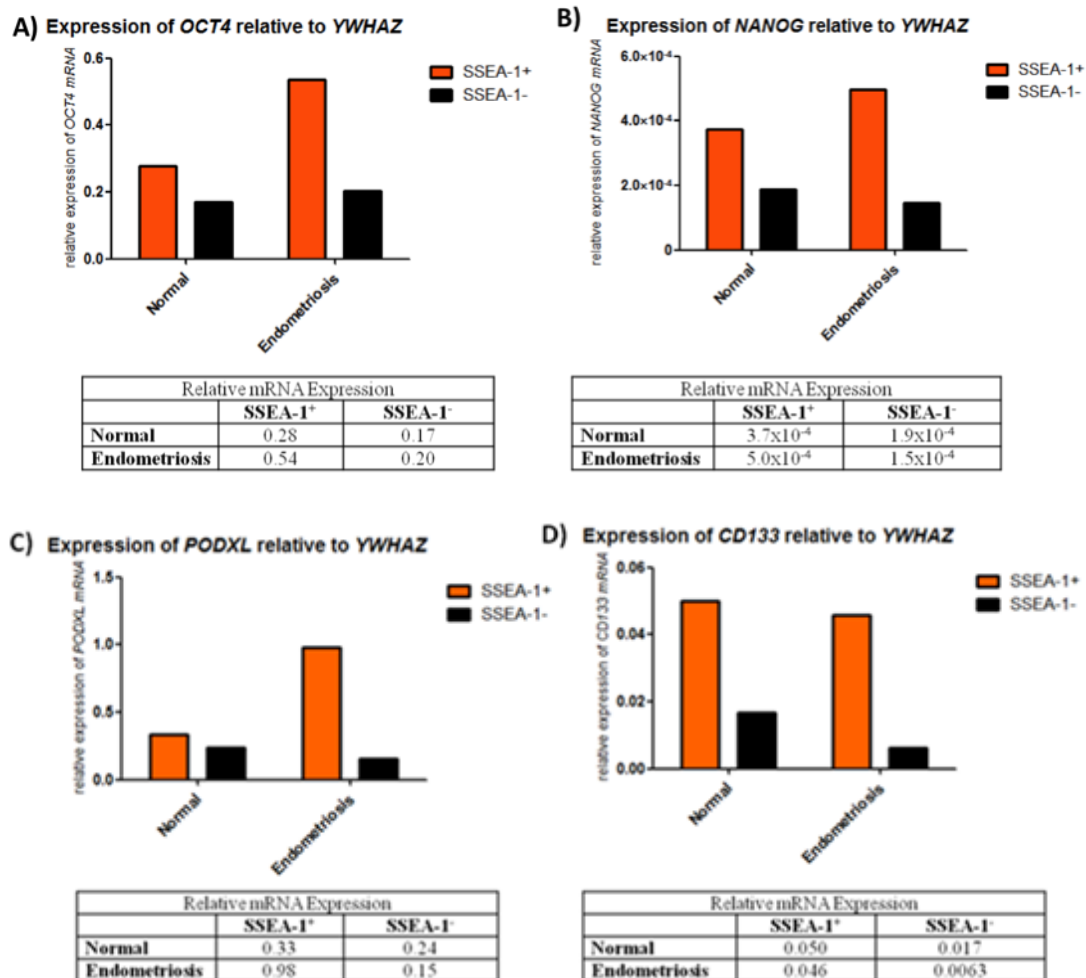
Figure 6.8 qPCR data of *A) OCT4 B) NANOG C) PODXL D) CD133* expression relative to the reference gene *YWHAZ* in SSEA-1 MACS sorted normal and endometriosis epithelial cells grown in 3D culture

Equal levels of *ERα* mRNA expression were measured in the SSEA-1^{+} and SSEA-1^{-} organoids grown from normal endometrium. However, when organoids were grown from cells taken from women with endometriosis, a large increase in *ERα* expression was clearly seen within the SSEA-1^{+} organoids. Similar differences in *PR* expression was seen between SSEA-1^{+} and SSEA-1^{-} organoids grown from the normal and endometriosis endometrium. When comparing the SSEA-1^{+} cells to SSEA-1^{-} population, the cells did not show a difference in *PR* mRNA expression after growth in

3D culture. This was in contrast to the significant up-regulation previously seen in the SSEA-1⁻ fraction when grown in 2D culture (see figure 6.9).

Relative mRNA Expression		
	SSEA-1⁺	SSEA-1⁻
Normal	0.042	0.047
Endometriosis	0.19	0.052

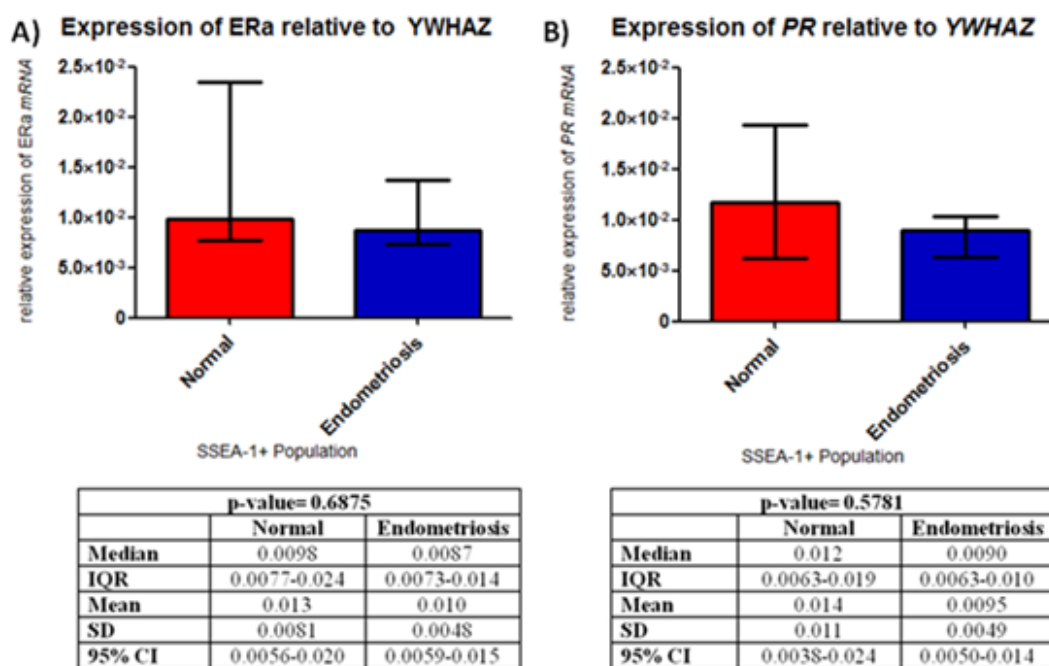| Relative mRNA Expression		
	SSEA-1⁺	SSEA-1⁻
Normal	0.015	0.10
Endometriosis	0.066	0.061
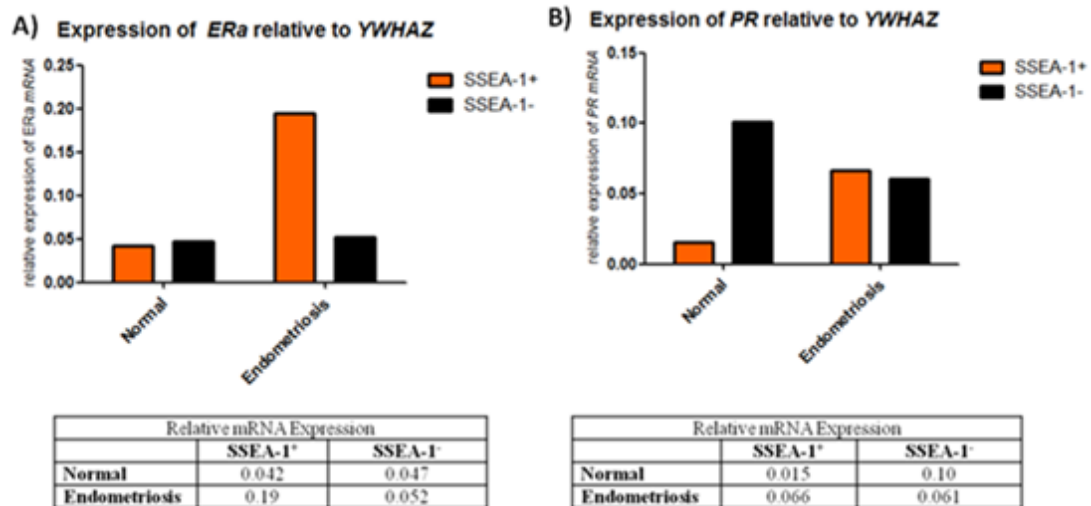
Figure 6.9 qPCR data of *A) ERα B) PR* expression relative to the reference gene YWHAZ in SSEA-1 MACS sorted normal and endometriosis epithelial cells grown in 3D culture

Like the stem cell markers, the expression of *FUT4* was seen to increase in the SSEA-1⁺ cells taken from the endometriosis patients in comparison to the SSEA-1⁺ cells grown from normal women in 3D culture. The difference between the expression of *CD9* in the SSEA-1⁺ and SSEA-1⁻ cells grown in 3D culture appeared to be more pronounced in the endometriosis group, but there was no obvious difference between the SSEA-1⁺ organoids between healthy and endometriosis groups in *CD9* expression. The difference in the expression of *FUT4* between the SSEA-1⁺ and SSEA-1⁻ organoids was also more pronounced in endometriosis (see figure 7.0).

A) Expression of *CD9* relative to *YWHAZ*

B) Expression of *FUT4* relative to *YWHAZ*

Relative mRNA Expression		
	SSEA-1+	SSEA-1-
Normal	25.64	16.52
Endometriosis	24.66	9.02

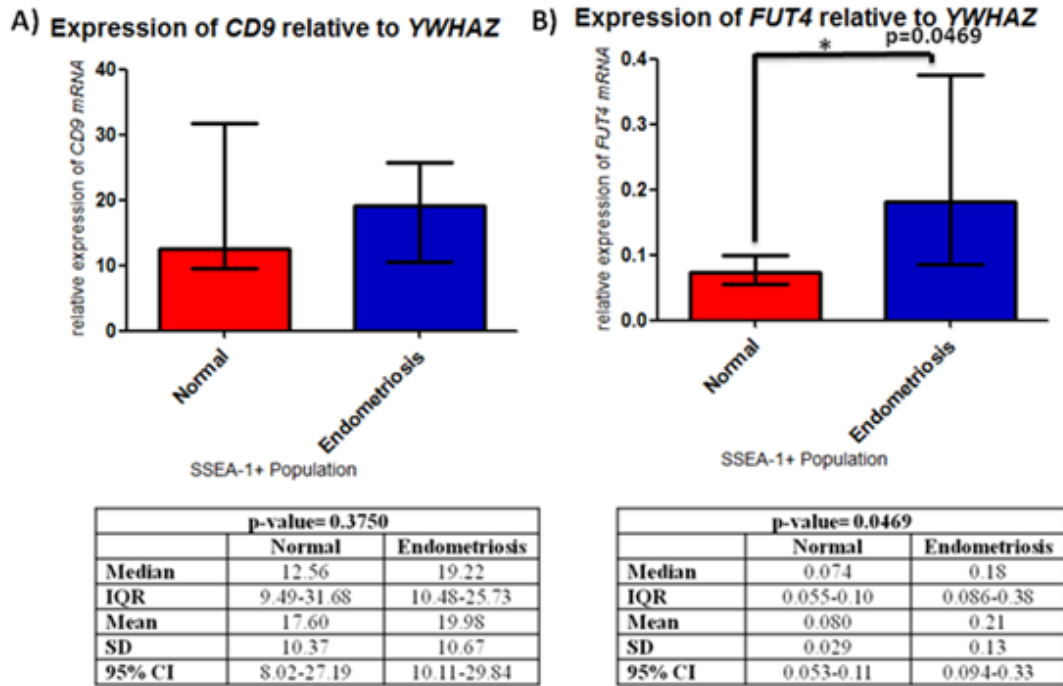| Relative mRNA Expression		
	SSEA-1+	SSEA-1-
Normal	0.15	0.093
Endometriosis	0.22	0.058
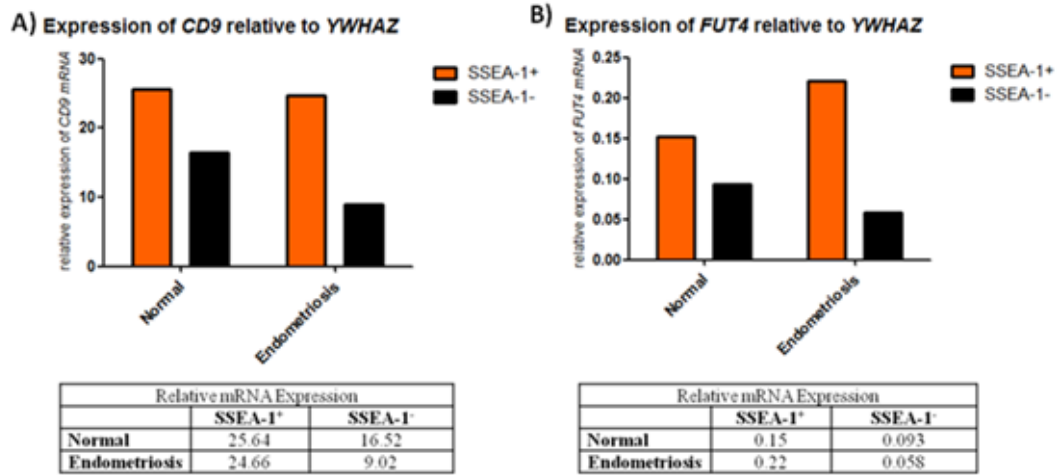
Figure 7.0 qPCR data of **A) CD9 B) FUT4** expression relative to the reference gene YWHAZ in SSEA-1 MACS sorted normal and endometriosis epithelial cells grown in 3D culture

4.5 Protein Analysis: Immunofluorescence (IF)

In order to see if an increase/decrease in mRNA levels of a particular target gene led to a noticeable increase/decrease in protein expression, IF staining was performed on the SSEA-1 MACS sorted normal epithelial cells plated onto 8-well chamber slides. However, the level of mRNA and the level of protein do not necessarily correlate (Gygi *et al*, 1999; Anderson *et al*, 1997). The images shown below represent IF staining for SSEA-1 within the MACS sorted SSEA-1⁻ and SSEA-1⁺ epithelial cell populations, and verify that there is depletion and enrichment for SSEA-1 in the respective fractions (figure 7.1).

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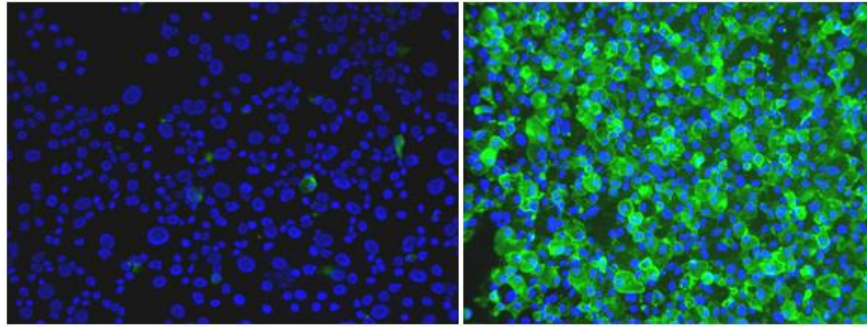
Figure 7.1 IF on MACS sorted SSEA-1⁻ (left) and SSEA-1⁺ (right) epithelial cells taken from the normal endometrium. Cells were cytocentrifuged onto glass slides, fixed and stained with anti-SSEA-1 (provided by Anthony Valentijn, Liverpool Women's Hospital)

One normal endometrial sample was mechanically and enzymatically digested, crudely sorted in to epithelial and stromal cells, and the epithelial fraction was grown in 2D culture for 5 days. The cells were then trypsinised, singly dispersed and MACS sorted on the expression of SSEA-1 in to SSEA-1⁺ or SSEA-1⁻ cells and both were plated on chamber slides for staining. Dual labelling was used to stain for cytokeratin (green fluorescence) and the protein of interest (red fluorescence). As DAPI was also used to stain the nuclei blue, any target nuclear proteins that were present were seen in pink due to the co-localisation of colours.

The protein for *OCT4* and *NANOG* was seen within both SSEA-1 populations, although the *OCT4* protein was seen at extremely low levels in only a few cells. This conflicted with qPCR data as unlike the protein, *NANOG* mRNA was consistently seen at a much lower level than *OCT4*. However, caution should be taken when comparing the expression levels of different genes with qPCR, as any differences in Ct values may be due to variations in primer efficiency rather than differences in gene transcription. In addition, nuclear staining patterns for these transcription factors appeared different to those seen in the human ESC positive controls, staining only part of the nucleus within the sorted cells rather than throughout the nucleus like

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the ESCs. Surprisingly *PR* and *PODXL* proteins were not found at all in either the SSEA-1 MACS sorted populations. The telomerase protein, unlike the *hTERT* mRNA, was present in many cells within both SSEA-1 sorted populations. This result correlates with previous telomerase activity data on these cells, and may highlight the difficulties of *hTERT* qPCR (Valentijn *et al,* 2013). The results confirmed the presence of *ERα* and *ERβ* protein in abundance in both SSEA-1 populations. This was in agreement with previous literature which has stated that although the *ERβ* protein is commonly seen, the mRNA is lowly expressed and does not correlate to the protein (Critchley *et al*, 2009) (see figures 7.2, 7.3, 7.4 and 7.4 continued below).
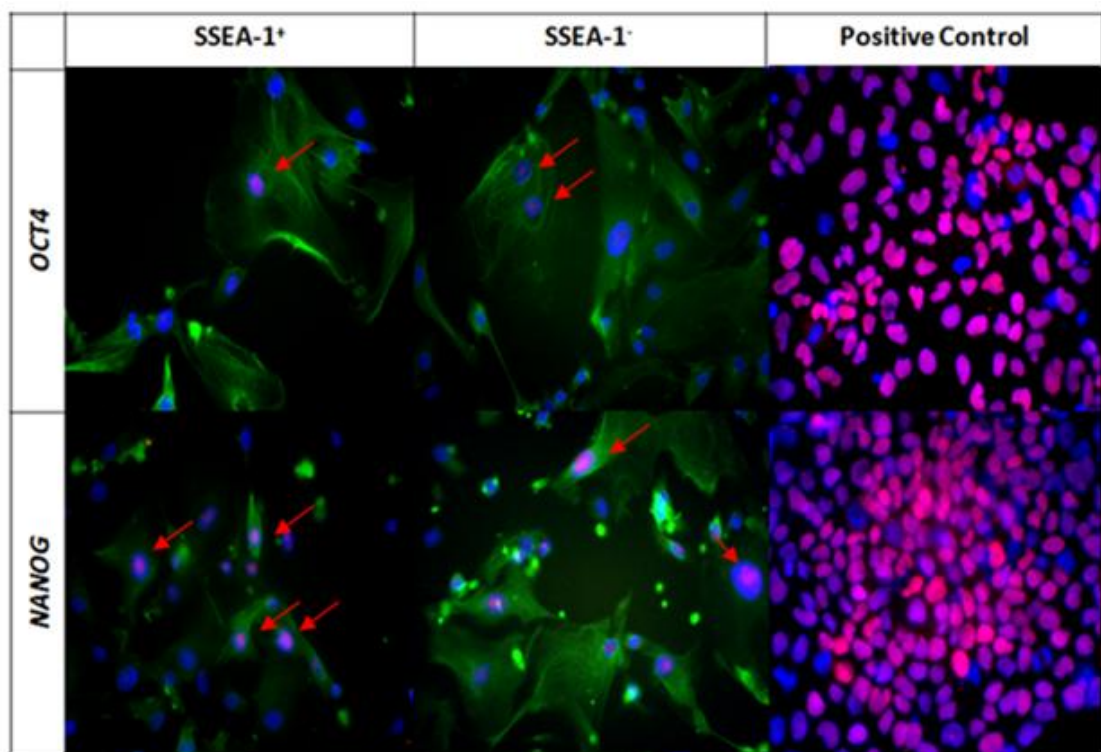
Figure 7.2 IF nuclear staining of *OCT4* and *NANOG* in SSEA-1 MACS sorted normal endometrial epithelial cells. Proportion of positively stained cells indicated by red arrows. Positive controls for *OCT4* and *NANOG*= human ESCs (x60 magnification)
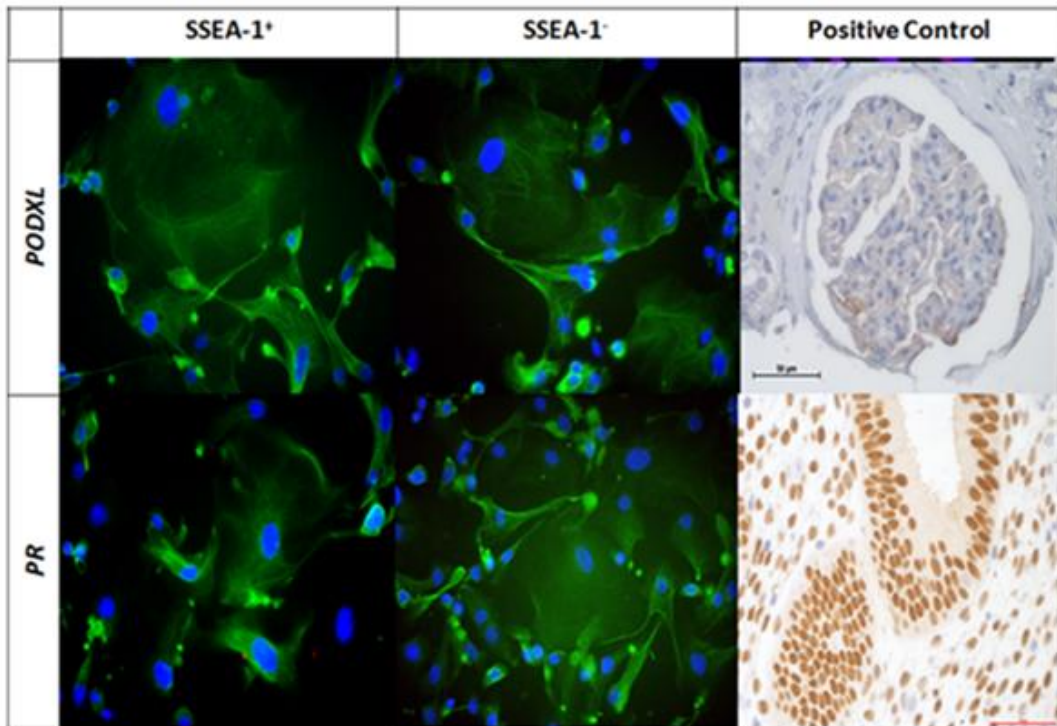
Figure 7.3 IF nuclear staining of *PODXL* and *PR* in SSEA-1 MACS sorted normal endometrial epithelial cells. Proportion of positively stained cells indicated by red arrows. IHC positive controls for *PODXL*= adult kidney; *PR*= normal endometrium (x60 magnification)
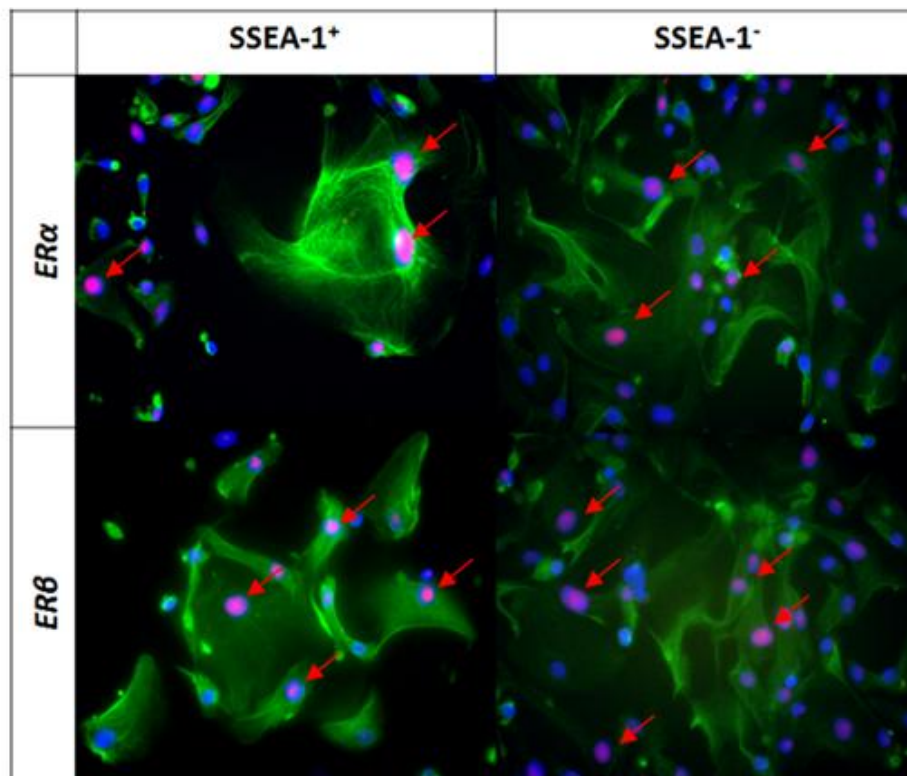
Figure 7.4 IF nuclear staining of *ERα* and *ERβ* in SSEA-1 MACS sorted normal endometrial epithelial cells. Proportion of positively stained cells indicated by red arrows (x60 magnification)
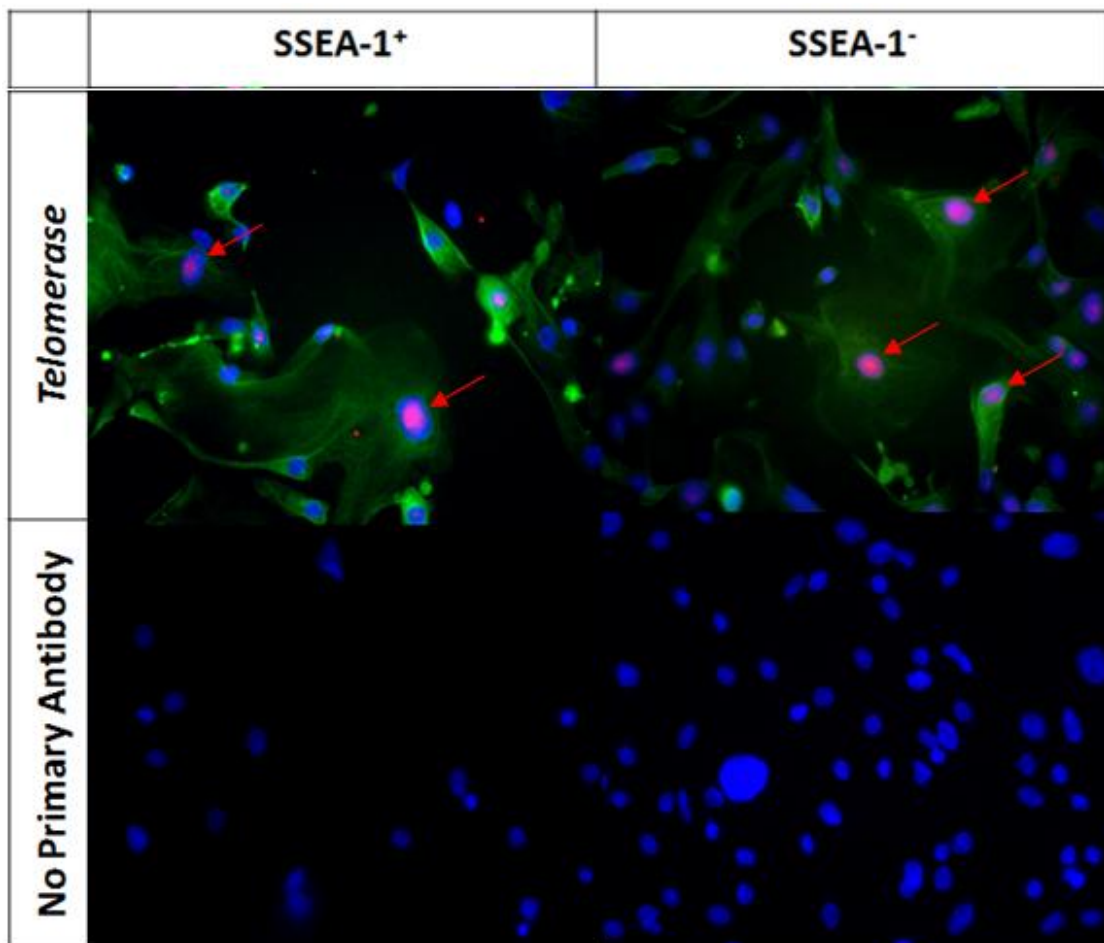
Figure 7.4 continued: IF nuclear staining of *telomerase* in SSEA-1 MACS sorted normal endometrial epithelial cells. Proportion of positively stained cells indicated by red arrows. No primary antibody used as negative control

As the *CD133* antibody that had previously been optimised within our laboratory had been discontinued, no *CD133* antibody was available within our laboratory to test on the SSEA-1 sorted cells within the time available. An image of IHC staining for *CD133* using the previous antibody has been included below to show the presence of this protein within endometrial cancer tissue (see figure 7.5).
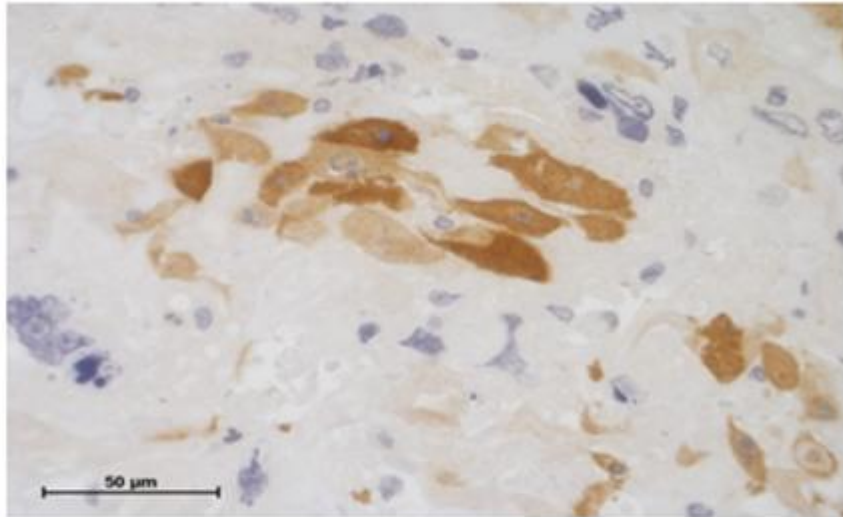
Figure 7.5 IHC staining on endometrial cancer tissue as positive control for *CD133* (x60 magnification). Positive cells stain brown (provided by Jo Drury, Liverpool Women's Hospital)

Chapter 5

Discussion

Chapter 5: Discussion

This was primarily a study examining the transcriptional profile of a selected set of genes in a sorted and isolated sub population of endometrial epithelial cells. The primary endometrial epithelial cells sorted on the expression of SSEA-1 are expected to contain a SPC population; therefore we evaluate the expression of genes that are associated with stem cell activity and an undifferentiated state. In order to further confirm that the SSEA-1$^+$ endometrial epithelial cells indeed have specific SPC activity, their gene expression profile for the same genes was compared to that of the SSEA-1$^-$ endometrial epithelial population, which represented more differentiated/mature epithelial cells from the endometrium. We studied the cells, cultured in both 2D and 3D culture systems to identify whether either of those conditions would favour the maintenance of an undifferentiated status. The gene expression profile of SSEA-1$^+$ cells was also compared in the cells derived from the endometrium of women with and without endometriosis to assess whether we could identify if they might play a role in the pathogenesis of this disease. IF staining of MACS sorted SSEA-1 epithelial cells was also used to see if any differences in gene expression also correlated with similar differences in the protein levels.

5.1 Normal Control Group

Our results confirmed the expression of human genes *OCT4, NANOG, PODXL, CD133, ERα, ERβ, PR, CD9* and *FUT4* across all samples included in our study, both SSEA-1$^+$ and SSEA-1$^-$ cell fractions. These results confirm that there is stem cell activity within the endometrial epithelial cell population, and supports the hypothesis that there may be cells with SPC activity in both epithelial and stromal fractions of the endometrium. *SOX2* mRNA was not detected within the full thickness endometrium or

either SSEA-1 epithelial cell fraction within any of the samples. Agreeing with this observation, the protein was also not visualised within the full thickness endometrium using IHC. This is consistent with the previous findings of Forte *et al* who analysed the expression profile of 13 genes, including *SOX2*, on non-sorted normal endometrial and endometriotic tissue using reverse transcription PCR. He found that *SOX2* expression was absent across all 14 endometrial tissue and 12 endometriotic tissues, within all phases of the menstrual cycle and all stages of endometriosis. The lack of *SOX2* may not be surprising, as although it may be needed for pluripotency in human ESCs, this is not an essential feature of ASCs and is unlikely to be necessary for the endometrial SPC (Forte *et al*, 2009). Unlike in the mouse ESCs, the key function of *SOX2* in human ESC has been described within the literature as largely dispensable compared to the other primitive pluripotency genes *OCT4* and *NANOG*, which are essential for the maintenance of human ESC self-renewal. Furthermore, evidence from induced pluripotent stem (iPS) cells suggest that the function of all these embryonic markers are rather different in human cells when compared to the mouse cells, and propose that the three pluripotency genes work independently to prevent differentiation along a specific lineage (Wang *et al*, 2012). *SOX2* and *SOX3* both repress mesendodermal differentiation, but the knock-down of *SOX2* within human ESCs does not seem to alter the pluripotent profile. This may be due to the compensatory action of *SOX3* which is up-regulated when *SOX2* levels are low, maintaining pluripotency within human ESCs (Wang *et al*, 2012).

Within the normal MACS sorted SSEA-1$^+$ cells grown in 2D culture, no significant differences in stem cell gene expression was seen between the SSEA-1$^+$ or SSEA-1$^-$ cells. This was evident in both MACS and FACS sorted samples, suggesting that both

populations possess cells expressing stem cell-specific genes. There are a number of possibilities which may account for these results. As MACS and FACS sorting are not 100% pure, any true differences in expression may have been masked by contamination of stromal stem cells or SSEA-1$^+$ epithelial cells within the SSEA-1$^-$ cell population. However, as FACS sorting has greater than 90% efficiency and the results from these samples support those seen from the MACS sorted samples, it is more likely that these results are a true reflection of gene expression profile within the populations studied. If SSEA-1$^+$ cells are largely a progenitor cell population, it is possible that a very small, more primitive stem cell sub-population is included within both SSEA-1$^{+/-}$ fractions.

In mouse ESCs, *OCT4*, *NANOG* and *SOX2* genes play a core regulatory function in maintaining pluripotency, however in human ESC, they may play a different role depending on their levels and the presence of other factors. For example, at high levels *OCT4* facilitates self-renewal only when *BMP4* is absent but specify mesendoderm in the presence of *BMP4*. Conversely, low levels of *OCT4* induce embryonic ectoderm differentiation in the absence of *BMP4* but specify extraembryonic lineages in the presence of *BMP4*. *NANOG* represses embryonic ectoderm differentiation but has little effect on other lineages, whereas *SOX2* and *SOX3* are redundant and repress mesendoderm differentiation. We have not studied the existence of *BMP4* in our cells. There are no conclusive reports on the *BMP4* expression in SSEA-1 expressing human endometrial glandular epithelium, and the existing single paper on *BMP4* expression in human endometrium only shows that in the secretory phase, endometrial epithelium does not express *BMP4* but stromal cells do (Wang *et al*, 2012; Stoikos *et al*, 2008). Therefore, we are unable to comment on the particular function of the expression of

these transcription factors in our cells. However, we can suggest that at the low levels of *OCT4* expression observed in endometrial epithelial cells *in vitro*, merely suggest epithelial differentiation of these cells. Another explanation may be the 2D culture system in which the sorted epithelial cells were grown, does not favour stemness but drives cells towards differentiation. Whilst traditional 2D culture may be sufficient to study the biological functions of endometrial cells, it has many limitations. When grown in 2D culture, endometrial cells lose their typical epithelial architectural/structural characteristics and their morphology from what is seen *in vivo*, and they no longer maintain their columnar shape and intercellular junctions, but become flat and lose their polarity. This may mean that the transcriptional stem cell profiles studied on the MACS and FACS sorted cells grown in 2D culture, may not be a true representation of the expression levels seen *in vivo* (Hai-yan *et al*, 2012).

We aimed to overcome this problem with the use of a 3D culture model; a system which is well recognised as the optimal method of studying endometrial epithelial cells *in vitro* (Hai-yan *et al*, 2012; Eritja *et al*, 2010; Bläuer *et al*, 2008). Matrigel is a gelatinous protein mixture urea extract derived from the basal lamina-rich mouse Engelbreth-Holm-Swarm (EHS) tumour. It is an ideal substrate for functional cells in *in vitro* as it reconstitutes the natural basal lamina and mimics the ECM within tissues *in vivo* (Hai-yan *et al*, 2012). 3D culture models using epithelial cells were first established using collagen-based matrices. However more recently, developments using breast epithelial cell lines have shown that cultures derived from EHS tumours are first choice for the growth of glandular epithelial tissues. This model retains structural polarity, epithelial cell morphology and is essential to imitate the architecture of the normal human endometrium *in vivo*. Nevertheless, although Matrigel is necessary for cellular polarity and glandular formation, it does not

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support glandular growth and proliferation. Additional supplements including epithelial EGF and ITS are required for the development of glandular structures, and therefore these were used within this study (Hai-yan *et al*, 2012; Eritja *et al*, 2010). Our data on gene expression from SSEA-1 sorted epithelial cells growth in 3D culture (or organoids), showed a clear difference from those grown on a 2D culture system, with an obvious up-regulation of all stem cell markers within the SSEA-1$^+$ organoids compared to the SSEA-1$^-$ organoids taken from the normal endometrium. This provides further evidence to support that the 3D culture system may be recapitulating the natural stem cell niche within the endometrium *in vivo*, hence preserving stemness of an endometrial stem cells and preventing differentiation of the SSEA-1$^+$ population. It is therefore more likely that the gene expression profile obtained from these gland-like structures is a more accurate representation of the levels that would be expressed from these cells within their natural microenvironment.

Expression levels of *OCT4* and *FUT4* mRNA were the highest of all genes throughout the study. *OCT4* consistently showed a level of expression considerably higher (approximately 1000 times higher) than that of its fellow transcription factor, *NANOG*. If the function of these markers is self-renewal and pluripotency as seen in ESCs, and with a similar function to NANOG, it was not anticipated that the signal level of *OCT4* would be so high in comparison within our adult tissue. Many papers are now being published stating that results of *OCT4* expression need to be interpreted with caution and much controversy has arisen over its existence within somatic cells (Lengner *et al*, 2008; Liedtke *et al*, 2008; Zangrossi *et al*, 2007; Seo *et al*, 2009). Although further evidence is now emerging to describe potential problems

when dealing with *OCT4*, many authors still have no knowledge of the recent discovery that two main variants of *OCT4* exist as a consequence of alternate splicing; *OCT4A* and *OCT4B* (also to referred to as splice variants 1 and 2. On a nucleotide level, both isoforms are identical from exon 2 through to exon 5. The genetic difference between the two variants arises from exon 1, which is missing from the *OCT4B* transcript and instead is replaced by 202 bps from the intron 1-2 region. Although similar in structure, the two variants have very different functions (Liedtke S, 2008). Unlike *OCT4A, OCT4B* has no function in regulating self renewal and cannot sustain stem cell properties (Seo *et al*, 2009). With IHC, *OCT4B* gives rise to staining within the cytoplasmic compartment, unlike *OCT4A* which is nuclear, raising question as to if *OCT4B* has any function as a transcription factor (Liedtke *et al*, 2008). In addition to these splice variants, at least 6 pseudogenes and other *OCT4-* like sequences, which are highly homologous for *OCT4A* exist. These account for yet more sources of potential difficulties in interpretation of the results. It is therefore crucial to verify that a primer set is *OCT4A* specific and discriminates this variant from other splice variants and pseudogenes to provide reliable mRNA data (Liedtke *et al*, 2008; Zangrossi *et al*, 2007). When entering our *OCT4* primer sequences into the Basic Local Alignment Search Tool (BLAST), it became evident that our primers are homologous with multiple splice variants and *OCT4*-like sequences (in addition to variants 1 and 2) and also pseudogenes 3 and 4. This may account for the high expression levels of *OCT4* seen within this study and so results should be interpreted with caution. Nevertheless, although not all of the *OCT4* signal may represent pluripotency or self-renewal, lower levels of *OCT4A* are still expressed by both SSEA-1 populations. Our *OCT4* primers were verified using Hues7 ESCs which displayed the same melting point and product size following qPCR and gel

electrophoresis as those seen within our sorted epithelial samples. In addition, IF staining using an *OCT4A* specific antibody showed positive staining in part of the nucleus, although at much low level and in far fewer cells when compared to the positive control of ESCs. This verifies that some of the SSEA-1 expressing epithelial cells do express *OCT4* and those cells may have SPC activity.

As SSEA-1 is a carbohydrate and therefore cannot be investigated directly using qPCR, *FUT4* was included within this study as the enzyme that catalyses its synthesis. It was therefore expected that the mRNA levels of *FUT4* would be significantly higher within the SSEA-1$^+$ endometrial epithelial cell fraction. Our results were somewhat surprising, showing that there was no difference in expression between the SSEA-1$^+$ and SSEA-1$^-$ fractions taken from the MACS sorted normal endometrial samples or endometriosis samples grown in 2D. In addition, FACS sorted samples taken from the normal endometrium showed a 2.5 fold up-regulation of *FUT4* within the SSEA-1$^-$ population when compared to the SSEA-1$^-$ cells. This could be explained by the fact that the cultured cells had changed their phenotype within 2D culture, and therefore expression levels of *FUT4* did not represent the true levels *in vivo*. When analysing the results collected from the sorted cells grown in 3D culture, which mimics their *in vivo* microenvironment, we can see that the expression levels reverse and *FUT4* levels are increased 1.7 fold in the SSEA-1$^+$ cells over the SSEA-1$^-$ cell fraction. Nevertheless, high *FUT4* expression within SSEA-1$^-$ epithelial cells suggests that this gene may have additional or alternative functions. On searching the publications, it has been suggested that other FucT genes may be involved in the synthesis of SSEA-1 also. Whilst the alternative name given to SSEA-1 by the National Center for Biotechnology Information (NCBI, 2012) is *FUT4*, there are a number of publications that suggest that *FUT9* is the more

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dominant enzyme responsible for its synthesis (Nishihara *et al*, 2003; Nakayama *et al*, 2001). SSEA-1 is regulated throughout brain development and acts as a cell-cell recognition molecule within the central nervous system. Nishihara *et al* compared the transcript levels of both *FUT9* and *FUT4* within developing brain, and found 15-100 times more *FUT9* transcript compared to *FUT4*. The synthesis of SSEA-1 throughout brain development was well correlated with that of *FUT9* (Nishihara *et al*, 2003). Nakayama *et al* also demonstrated that *FUT9* has 20-fold stronger activity for the synthesis of SSEA-1 over *FUT4* within mature granulocytes (Nakayama *et al*, 2001). Sialylated SSEA-1 or Le^x (sLe^x) is a fucosylated structure related to SSEA-1. Various studies have shown that multiple FucTs including *FUT3, FUT5, FUT6* and *FUT7* may all be involved in its synthesis (Liu *et al*, 2008; Nordén *et al*, 2009). This evidence suggests that there may be other important factors in addition to *FUT4* which control the synthesis of SSEA-1. Furthermore, the *FUT4* expression in the normal endometrium is maximum in the functionalis during the secretory phase of the cycle where SSEA-1+ epithelial cells are not present (Ponnanpalam *et al*, 2008; Valentijn *et al*, 2013), suggesting that FUT4 is not co-expressed with SSEA-1 in endometrial epithelial cells.

The results obtained from *ERα* and *PR* transcripts between MACS and FACS SSEA-1 sorted normal endometrial cells, showed obvious differences between the expression levels in the two populations, with a clear up-regulation within the SSEA-1$^-$ cells. The expression of these cells are likely to be present in terminally differentiated endometrial epithelial cells and more primitive, undifferentiated epithelial cells are unlikely to be either steroid hormone responsive and may not express the receptors for them. This reached significance for both *ERα* and *PR* (WSR

test; p=0.0156) within the MACS sorted cells, and indicates a more differentiated cell population within the SSEA-1⁻ fraction. This may not have reached the level of significance within the FACS sorted samples due to the small number of samples used in comparison (n=3), or because contaminating stromal cells within the SSEA-1⁻ MACS sorted samples were expressing these genes also. Within 3D culture, the difference in *PR* levels was more pronounced, with a 6.7 fold up-regulation of *PR* within the SSEA-1⁻ fraction. Although this was not the case with *ERα* which displayed very similar levels of expression within both fractions grown in 3D culture, we know that *PR* is a marker of a more terminally differentiated cell state than *ERα* and therefore still supports the previous findings (Prianishnikov *et al*, 1978).

IF staining was used to verify the presence of the protein translated from each of these transcripts, within SSEA-1 MACS sorted cells taken from one patient with a normal endometrium. The transcription factors *OCT4A* and *NANOG* showed nuclear staining within a selected number of cells within both SSEA-1⁺ and SSEA-1⁻ cells, the correct location for both proteins. More cells stained positively for *NANOG* than *OCT4A,* which acts as more evidence to suggest that the *OCT4* primer pair did not exclusively amplify *OCT4A* mRNA. Interestingly, the *OCT4* and *NANOG* positively stained cells showed a different expression pattern to the ESC positive control. Within the ESCs, staining for both proteins were seen throughout the nuclei, however only part of the nuclei were stained for these proteins within the sorted endometrial epithelial cells. *ERα*, *ERβ* and telomerase showed nuclear staining in a high proportion of cells in both of the sorted populations. For *ERβ* and telomerase, this contradicted what was detected via qPCR, as mRNA levels for both these genes were below the level of detection. These results support the claims made by Lehner *et al* and Critchley *et al* as described previously in chapter 3 (Lehner *et al*, 2002;

Critchley *et al*, 2002). In contrast, *PR* and *PODXL* which were both highly amplified using qPCR, showed negative staining for protein across all sorted cells using IF. Although it is generally assumed that mRNA and protein levels for a particular gene should correlate, analyses in yeast and mammalian cells have shown that this is not necessarily the case (Gygi *et al*, 1999; Anderson *et al*, 1997). In fact, studies have now proven that mRNA levels alone act as unreliable predictors for corresponding protein. Protein levels are dependent on a number of factors other than the rate of transcription, including nuclear export of the transcript, mRNA localisation, transcript stability, translational regulation and protein degradation or *in vivo* half life. Once translated, proteins may also go through post-translational modifications, for example glycosylation or phosphorylation, or may undergo proteolytic cleavage (Greenbaum *et al*, 2003; Pradet-Balade *et al*, 2001). These processes are still not sufficiently defined and may account for the discrepancies between the mRNA and protein abundances seen within our study. For example, if *PR* and *PODXL* proteins have short half lives, transcription may occur at high rates due the high demand and fast turnover. On the other hand, *ERα* and telomerase may be stable proteins requiring much lower rates of transcription (Greenbaum *et al*, 2003; Pradet-Balade *et al*, 2001).

5.2 Endometriosis Group

As endometrial SPCs are thought to be involved in the pathogenesis of endometriosis, patients suffering with this disease were also included in our study. Similar to the results obtained from the control patients, no significant differences in *OCT4, NANOG, PODXL* and *CD133* mRNA levels were found between the two human epithelial cell populations MACS sorted on SSEA-1 and grown in 2D, taken from endometriosis patients. When placed in 3D culture representing their *in vivo*

environment however, similar to the normal endometrium, these genes were clearly up-regulated within the SSEA-1$^+$ population when compared to the SSEA-1$^-$ cells. In addition, these differences in expression appeared to be more exaggerated in those with endometriosis than those with normal endometrium, suggesting more stem cell activity within these cells from the pathological endometrium. When comparing the MACS sorted SSEA-1$^+$ epithelial cell populations directly between the normal and endometriosis patients, statistical differences were seen with significant up-regulation of *OCT4* and *NANOG* within patients with endometriosis. All of these results suggest that the SSEA-1$^+$ population have heightened stem cell-like activity in endometriosis and that they may be involved in the pathogenesis of this condition. If a subpopulation of the SSEA-1$^+$ epithelial cells are SPCs, then these results would support the widely accepted theory that endometrial SPCs are involved in the aetiology of this condition (Figueira *et al*, 2011; Gargett *et al*, 2010).

In the patients suffering from endometriosis, *ERα* showed a 1.7 fold higher level of expression in the SSEA-1$^-$ fraction compared to the SSEA-1$^+$ cells. Unlike the significant up-regulation of *ERα* seen previously within the normal SSEA-1$^-$ cells, this level of expression was not significant for those with endometriosis. In addition, when growing these sorted cells in 3D culture that mimics their natural environment, an apparent up-regulation of *ERα* was seen within the SSEA-1$^+$ cells taken from patients with endometriosis compared to the paired SSEA-1$^-$ cells grown in 3D culture and to the SSEA-1$^+$ cells from the normal healthy endometrium. Collectively these results may be explained by the increase of local oestrogen levels seen within patients with endometriosis (Gurates *et al*, 2003). Endometriosis is recognised as an 'oestrogen responsive disorder', and the growth and maintenance of ectopic

endometriotic lesions is dependent on the action of oestrogen. In the endometrium, oestrogen acts as a potent mitogen and it is the increased levels of cellular aromatase expression in these patients with endometriosis and that leads to greater local oestrogen production compared to healthy individuals (Gurates *et al*, 2003). SSEA-1^+ cells are evidently more sensitive and responsive to *ERα* within patients with endometriosis, again substantiating their role in the pathogenesis of endometriosis and their existence as endometrial SPCs.

PR showed a significant up-regulation in mRNA levels within the SSEA-1^- population compared to the SSEA-1^+ cells within patients with endometriosis. This is similar to the results obtained from sorted cells taken from the normal endometrium grown in both 2D and 3D culture systems, and again seems to indicate that a more differentiated cell type may lie within the negative fraction. Although the SSEA-1^+ population grown in 3D culture showed higher levels of *PR* in the endometriosis samples compared to the control samples, this level of expression was no different to that seen within their SSEA-1^- counterpart. In the literature, it is now widely accepted that endometriosis is physiologically associated with an element of 'progesterone resistance' and lower overall levels of total *PRs,* therefore explaining the equal levels of expression seen in our results. This phenomenon most probably occurs to counteract the anti-proliferative and differentiative effects that progesterone has on endometrial cells, thereby contributing to the maintenance of the disease. Several mechanisms have been suggested to explain why this resistance process occurs. Some claim that there is altered expression of both isoforms of *PR* (with a complete lack of *PR-B),* their chaperone proteins such as FKBP52 and co-regulators including HIC-5/ARA55. Other theories suggest that there is activation of pro-

inflammatory transcription factors which compete with *PR* over limited co-regulators, and also that an increase in free radical production and oxidative stress signals may lead to post-translational alteration of *PRs* (Attia *et al*, 2000; Brosens *et al*, 2012; Bulun *et al*, 2006).

Another interesting finding in our study was related to the endometrial epithelial cell marker *CD9*. As a widely accepted marker for endometrial epithelial cells, it was expected that the levels of expression for *CD9* would be equal in both of the SSEA-1 sorted populations. This was the case within all samples grown in the 2D culture system, as no significant differences in expression levels were found between the two populations in the control of endometriosis patients. However, when placed in 3D culture, up-regulation of *CD9* was evident within the SSEA-1$^+$ population compared to the SSEA-1$^-$ cells; with a 1.5 fold increase within the normal samples and 2.8 fold within the patients with endometriosis. *CD9* is found to be highly expressed in murine and human ESCs and is decreased shortly following differentiation. It is likely that *CD9* may be under the regulation of the LIF/STAT3 mouse ESC pathway, which is required for the self-renewal of undifferentiated mouse ESCs. *CD9* is also expressed in some ASC populations including HSCs where it is found to be important for maintenance of the population and colony formation (Oka *et al*, 2002; Akutsu *et al*, 2009; Aoyama *et al*, 1999). If this is the case, then *CD9* may have a role in maintaining stemness within the endometrium also. This would account for the up-regulation seen in the normal SSEA-1$^+$ population when grown in an environment that represents the stem cell niche, and would account for the greater difference seen within patients with endometrioisis which is associated with abnormal stem cels. Also this may simply mean greater survival of the epithelial

cells in the SSEA-1$^+$ population grown in 3D, and it may be possible that the SSEA-1$^-$ epithelial cells do not survive as much in 3D culture. If more stromal cells are present in the SSEA-1$^-$ population after 2 weeks in 3D, there will be a reduction in *CD9*. We have not tested this theory by also looking at the changes in a gene specific to stromal cells such as *CD10/CD13* in our study, which may have helped us to confirm the reasons for the observed results.

As previously mentioned, in the 2D culture samples, no differences in *FUT4* expression was seen between the SSEA-1$^+$ and SSEA-1$^-$ populations taken from normal or endometriosis patients. It is highly possible that other FucT enzymes may be responsible in the synthesis of SSEA-1 also. However, when comparing *FUT4* expression between the SSEA-1$^+$ populations isolated from normal and endometriosis patients, a significant increase in expression was seen in the cells from women with endometriosis. In addition, when placed in 3D culture, expression of *FUT4* was greater in the SSEA-1$^+$ cells of the normal samples and even more so in the endometriosis samples when compared to the paired SSEA-1$^-$ fraction. This provides further evidence to suggest that *FUT4* is at least partly associated with the synthesis of SSEA-1, and therefore transcript levels were raised within the SSEA-1$^+$ cells grown in 3D culture, where cell proliferation was stimulated. Transcript levels were significantly higher in SSEA-1$^+$ cells taken from endometriosis patients, supporting the hypothesis that SSEA-1$^+$ cells are activated in endometriosis and are involved in its aetiology.

5.3 Limitations of the Study

Throughout the course of this study, a number of limitations were encountered which needed to be considered and acknowledged when interpretations are made for our data. One of these limitations was the difficulty of obtaining samples with a good RNA quality and yield for use within qPCR. Due to large variations between samples, not all biopsies collected would give rise to a good epithelial cell yield suitable for MACS/FACS sorting. In addition, cells grown in 3D culture gave rise to small clusters of cells producing very little RNA and therefore these samples had to be pooled. This is not the ideal sample for study in one hand, but may provide some information that can be related to the population of patients included in general. Due to the limited number of suitable samples and also the inaccessibility of the FACS machine, the number of samples which could be studied within the time available was also limited. Ideally, larger sample sizes (especially for FACS and 3D culture analysis) would have provided more robust and reliable data.

Another limitation that must be considered is variation between the samples themselves. Two different techniques of sample collection, pipelle and full thickness, were implemented to collect endometrial biopsies within this study according to the patient availability and the kind of surgical procedures they were undergoing. It is commonly accepted that pipelle sampling only extracts the cells which are located within the functional layer and not those within the basalis, although cells from the basal layer may still be taken up through this gentle method of suction. As it is hypothesised that endometrial SPCs are found primarily within the basal layer of the endometrium, this raises the question as to whether the results obtained from pipelle samples were an accurate representation of the SPC population. Due to the difficulty in

obtaining samples however, this could not be avoided but must be considered when interpreting the results, especially if the number of pipelle/full thickness samples were unevenly distributed between the groups.

Due to the difficulties encountered when trying to obtain suitable samples, biopsies were taken from women within all stages of the menstrual cycle. Different cycle stages may be associated with different levels of SPC activity, for example an increase in the proliferative phase. An uneven distribution of samples taken from different stages of the cycle between the normal and endometriosis groups may therefore raise question as to the comparability of the results between the groups. Nevertheless, due to the limited number of samples, the individual groups could not be subdivided further and analysed according to cycle phase. Although it could be argued that as all cells were cultured in same in vitro conditions prior to gene analysis, their phenotype would no longer reflect their original cycle stage. On the other hand since they do express some ovarian steroid receptors, they are likely to have some responsiveness to these hormones in vivo, so samples from different stages of the cycle may have SPCs with different activation status.

5.4 Future Directions

In order to encourage scientific advances and improvement within this field, suggestions should be made for future work. The obvious need is to increase in the number of patients and samples included in this study to confirm the results and improve the robustness and reliability of them. Specifically, the study of more FACS sorted samples for both normal end endometriosis would fully validate our results, as FACS produces more pure populations of cells than MACS. Our study specifically

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lacked a good number of samples to be grown in 3D Matrigel to provide statistically robust data. With more samples, this would have been possible. Ideally, these gland-like structures too should be grown from FACS sorted cells. This has been attempted within our lab but FACS sorting affects the function and viability of these primary epithelial cells to a greater degree than MACS sorting, therefore the cells do not seem to grow following the FACS sorting. It may be speculated that the FACS sorting process damages the cells, for example due to the exposure to high pressures or a prolonged time in suspension, therefore affecting their growth and viability. Although less efficient, MACS sorting is far gentler and growth of the cells post-sort is not affected. With an increase in the sample size included within this study, patients could also be subdivided based on the phase of their menstrual cycle, and results could be compared according to cycle phase and endometriosis stage to assess whether there may be any other correlations.

As previously mentioned, one important property of stem cells which must be investigated is their potency. The differentiation capacity of the SSEA-1 sorted epithelial cells should be assessed for multi-lineage differentiation potential when cultured under the correct conditions, including osteogenic, adipogenic and chondrogenic differentiation. Studies to explore *in vivo* tissue reconstitution of the SSEA-1$^+$ population and the responsiveness of the gland-like structures to ovarian hormones, progesterone and oestrogen, will provide more evidence to suggest their behaviour as endometrial SPCs and further our understanding about this population of cells. Once SSEA-1 is firmly established as a marker that selects for a population containing the endometrial epithelial SPC population, research needs to be invested into identifying a secondary surface marker that co-localises with SSEA-1. It is

unlikely that all epithelial cells that express SSEA-1 are SPCS and therefore a secondary marker would further select for the subpopulation within the SSEA-1$^+$ cells of specific endometrial epithelial SPCs.

5.5 Conclusion

In conclusion, this study has provided data on specific gene expression profile of an endometrial epithelial sub-population characterised on the expression of the surface marker SSEA-1. The genes that were expressed in this population when compared to the SSEA-1$^-$ cells have provided evidence to suggest that SSEA-1$^+$ population may have a subset of cells with SPCs activity in the human endometrium. Results have shown that genes associated with increased stem cell activity and a more undifferentiated cell state are expressed in the SSEA-1$^+$ endometrial epithelial cell population when compared to its paired SSEA-1$^-$ counterpart, but only when placed in the correct conditions that mimic their *in vivo* micro-environment. This study has demonstrated the importance of epithelial cells in particular to be grown in 3D culture and the importance of the stem cell niche as a functional unit. We have also shown that a subset of these SSEA-1$^+$ endometrial epithelial cells may be involved in the pathogenesis of endometriosis, observing pronounced expression of genes that may induce stem cell activity, oestrogen responsiveness and progesterone resistance in this condition. Thus our study provides further evidence to suggest that endometrial SPCs are involved in the aetiology of endometriosis.

As not all SSEA-1$^+$ endometrial epithelial cells but only a small subset within the SSEA-1+ population will be SPCs, it is important to find another surface marker to select a pure subpopulation of SPCs to assess whether they possess all known functional SPC activity in functional assays such as self-renewal, clonogenicity,

differentiation potential and *in vivo* tissue reconstitution potential. These experiments needed to be performed on these cells before any conclusions are made, however, growth of primary endometrial epithelial cells are reputed to be challenging and usually they do not withstand separation in to single cells. Therefore it will be a challenging task to perform these assays in the future studies. Also additional investigations to evaluate the responsiveness of these cells to steroid hormones will also provide valuable information of the in vivo endometrial regeneration which is regulated by these hormones.

Chapter 6

Appendix

Appendix I: References

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Appendix II: Specification List for Reagents and Equipment

Table 18 List of reagent specifications

Reagents	Company, City, Country	Catalogue Number
2-propanol	Sigma-Aldrich, Dorset, UK	I9516
5x 1st strand buffer	Invitrogen, Paisley, UK	P/N y02321
5x DNA loading buffer	Bioline, London, UK	BIO-37045
Agarose, Molecular grade	Bioline, London, UK	BIO-41025
Anti-SSEA-1(CD15) MicroBeads	Miltenyi Biotec, Surrey, UK	# 130-090-101
BD Matrigel™	BD Biosciences, Oxford, UK	#354234
Bovine Serum Albumin (BSA)	Sigma-Aldrich, Dorset, UK	A9418
Chlorhexidine gluconate 0.05% (Sterets Unisept)	Medlock Medical Ltd, Oldham, UK	AA161/01701
Chloroform	Sigma-Aldrich, Dorset, UK	C2432
Collegenase	Invitrogen, Paisley, UK	#17018029
Dispase	Invitrogen, Paisley, UK	#17105041
Dithiothreitol (DTT) (0.1M)	Invitrogen, Paisley, UK	P/N y00147
DMEM/F12	Sigma-Aldrich, Dorset, UK	D6421
DNase	Roche, Germany	#11284932001
dNTP Set	Bioline, London, UK	BIO-39025
Epithelial growth factor	Sigma-Aldrich, Dorset, UK	E9644
Ethanol	Sigma-Aldrich, Dorset, UK	E7023
Ethidium bromide	Molecular Sigma Biology, Dorset, UK	E-7637
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, Dorset, UK	E-5134
Fetal Bovine Serum (FBS)	Biosera, East Sussex, UK	FB-1370/100
Ficoll	GE Life sciences, Little Chalfont, UK	17-1440-02
FlashGel® DNA Marker (100bp-4kb)	Lonza, Slough, UK	50472
FlashGel® Quantladder (100bp-1.5kb)	Lonza, Slough, UK	50475
FlashGel™ Loading Dye (5x)	Lonza, Slough, UK	50463
Glacial Acetic Acid (17.M)	BDH, London & Bristol, UK	UN2789
Glycogen	Invitrogen, Paisley, UK	10814-010
Insulin-transferrin-selenite (ITS)	Invitrogen, Paisley, UK	#41400-045
KAPA SYBR FAST qPCR Master Mix (2x)	Kapa BioSystems, Boston, US	KK4602
$MgCl_2$ 100mM	BDH, Leicestershire, UK	
Mounting Medium for Fluorescence with DAPI	Vector Laboratories, Peterbrough, UK	H-1200
NaCl	Sigma-Aldrich, Dorset, UK	S5886-500G

Table 18 continued List of reagent specifications

Reagents	Company, City, Country	Catalogue Number
Neutral Buffered Formalin (NBF) 10%	Sigma-Aldrich, Dorset, UK	HT501320-95L
Normal goat serum (NGS)	Biosera, East Sussex, UK	GO-605
Nuclease free water	Sigma-Aldrich, Dorset, UK	P4417-100TA
Phosphate Buffered Saline (PBS)	Sigma-Aldrich, Dorset, UK	W4502
Primocin	Invitrogen, Paisley, UK	P4417-100TA
Random Hexamers (100ng/μl)	Qiagen, West Sussex, UK	#ant-pm-2
RQ1 DNase Buffer	Promega, USA	AM7020
RQ1 DNase Stop Soln	Promega, USA	M198A
RQ1 DNaseI	Promega, USA	M1994
SuperScript III (200U/μl)	Invitrogen, Paisley, UK	M610A
Triton 0.2%	BDH, Leicestershire, UK	18080-044
TRIzol® reagent	Invitrogen, Paisley, UK	9002-93-1
Trypsin/EDTA solution	Sigma-Aldrich, Dorset, UK	15596-026
Tween	BDH, Leicestershire, UK	#T4049

Table 19 List of equipment specifications

Equipment	Company, City, Country	Catalogue Number
Carbon steel surgical blade, size 22	Swann-Morton, Sheffield, UK	0205
FACSAriall cell sorter	BD Biosciences, Oxford, UK	
FACSCalibur cytometer	BD Biosciences, Oxford, UK	342975
FlashGel dock	Lonza, Slough, UK	57025
Flashgel System	Lonza, Slough, UK	57067
FlashGel™ Camera	Lonza, Slough, UK	57040
Hand-held homogenisor	Qiagen, West Sussex, UK	9001272
Molecular Imager® Gel DocTM XR	Bio-Rad, Hertfordshire, UK	#170- 8270
Nanodrop software	NanoDrop Technologies, Wilmington, USA	ND-8000
NanoDrop™ 1000 Spectrophotometer	NanoDrop Technologies, Wilmington, USA	ND-1000
Rotor-Gene 3000 centrifugal real-time cycler	Corbett Research, Sydney, Australia	
Teales Vulsellum uterine forceps	Phoenix Surgical Instruments Ltd, Hertfordshire, UK	PH725903

Table 20 List of consumable product specifications

Equipment	Company, City, Country	Catalogue Number
8-well chamber slides	BDH Chemicals, Leicestershire, UK	734-2050
22x40mm coverslip	WBFM, London, UK	GP1001125004
24-well tissue culture plate	Biowhittaker, Suffolk, UK	DMEM-F12
Cell strainer, 40μm	Miltenyi Biotec, Surrey, UK	# 130-090-101
Centrifuge tube, 15ml	Appleton Woods, Birmingham, UK	SC060
Chamber removal tool	BDH chemicals, Leicestershire, UK	734-0089
Falcon tube, 50ml	BD Biosciences, Oxford, UK	358206
MACS separation column	Miltenyi Biotec, Surrey, UK	MS columns, #130-042-201
Microcentrifuge tube, 1.5ml	Sarstedt Ltd, Leicester, UK	72699
Parafilm	Pechiney Plastic Packaging, Menasha	WI 54952
Pastette, 1ml	StarLab, Milton Keynes, UK	#E1414-0110
PCR tube, 0.2ml (flat cap)	StarLab, Milton Keynes, UK	I1402-8100
Petri dish, 100mm	BDH chemical, Leicestershire, UK	734-2321
Tissue cultureT75cm^2 flasks	BD Biosciences, Oxford/Swindon/Plymouth, UK	137787
Two-tier 16+1 2.2% agarose cassette	Lonza, Slough, UK	57032
Universal tube, 100ml	Sarstedt Ltd, Leicester, UK	
Universal tube, 30ml	Sarstedt Ltd, Leicester, UK	
Universal tube, 50ml	Sarstedt Ltd, Leicester, UK	
Yellow pre-filter, 30μl	Miltenyi Biotec, Surrey, UK	#130-041-407

Appendix III: LREC Approval

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05 October 2009

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

Dear Dr Hapangama

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

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

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

Confirmation of ethical opinion

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

Ethical review of research sites

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

Conditions of the favourable opinion

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

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

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

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

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

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

Approved documents

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

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

Statement of compliance

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

After ethical review

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

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

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

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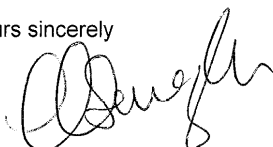- Progress and safety reports
- Notifying the end of the study

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

changes in reporting requirements or procedures.

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

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

Yours sincerely

Professor Sobhan Vinjamuri
Chair

Email: carol.ebenezer@northwest.nhs.uk

Enclosures:	List of names and professions of members who were present at the meeting and those who submitted written comments
	"After ethical review – guidance for researchers"
Copy to:	Mrs Gillian Vernon

National Research Ethics Service

North West 2 Research Ethics Committee - Liverpool Central

3rd Floor
Barlow House
4 Minshull Street
Manchester
M1 3DZ

Tel: 0161 625 7818
Fax: 0161 237 9427

22 March 2010

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

Dear Dr Hapangama

Study title:	**Telomere Length, Expression of Telomerase and hTert in Normal and Pathological Endometrium**
REC reference:	**04/Q1505/112**

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

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

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

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

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

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

Yours sincerely

[signature]

C. Ebenezer (Mrs)
Committee Co-ordinator

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

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

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

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

Liverpool Women's
NHS Foundation Trust

14th April 2011

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

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

Dear Dharani

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

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

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

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

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

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

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

Yours sincerely

Gillian Vernon
Research & Development Manager

Liverpool Women's

Appendix IV: Patient Information Leaflets

Liverpool Women's
NHS Foundation Trust

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.

Hapangama / Stem cells Version 1(revision 1)   **Date 09/09/09**

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.

Ethics Submission No: 09/H1005/55
PATIENT INFORMATION SHEET
"Endometrial stem cell Study"
Role of endometrial stem cells in endometriosis.
Version 2: Biopsy of Endometrium and endometriosis lesions

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

Thank you for reading this

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

What are stem cells?
Stem cells are special cells that can renew themselves (adult stem cells). The inner-lining of the womb (endometrium) has these stem cells that are likely to be responsible for its monthly regeneration. With monthly bleeding these cells are shed and can be expelled into the abdominal cavity. If these cells are implanted in the pelvis they can cause endometriosis as endometriosis occurs when endometrial cells of are found growing outside of the womb. We believe that abnormalities of these stem cells may cause endometriosis.

Why have I been chosen?
We are looking for a total of 80 women with endometriosis (you must have been off all hormonal contraception for at least 3 months), who have regular periods. If you have endometriosis and is scheduled to have surgery to remove endometriosis lesions we will ask you if you would want to take part in the study.

Do I have to take part?
It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?
1. *If you are having a hysterectomy:*

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Your operation will continue exactly as planned. However, once the operation is over, a small sample of the womb that has already been removed will be taken for the research.

2. *If you are not having a hysterectomy:*

Your operation will continue exactly as planned. However, a pipelle sample (see below) will be taken from you. Once the operation is over, a small sample of the endometriosis tissue that has already been removed will also be taken for the research.

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

How is the endometrial pipelle sample done?

Whilst you are under anesthetic, the doctor will place a speculum (just like when you have a cervical smear) in the vagina. A plastic instrument (like a blunt drinking straw) will then be introduced through the neck of your womb to gently suck some cells from the inner lining of the womb. These cells will be then sent to the laboratory to be examined. This procedure is routinely done in our Gynaecology clinic and apart from the mild lower abdominal period like discomfort and vaginal spotting, it does not usually cause other problems. Since you are going to have the biopsy taken at the time of the operation under anesthetic, you will not have any extra discomfort.

What are the possible benefits of taking part?

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

What if something goes wrong?

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

Data management

The information gathered from this study will be entered into a computer database and will be analyzed in a strictly confidential manner, in compliance with the Data Protection Act. Regulatory authorities for approving medicines and the University of Liverpool may wish to look at medical records to check that the study has been performed correctly. All information, which is collected about you during the course of the research will be kept strictly confidential. Any information about you, which leaves the hospital, will have your name and address removed so that you cannot be recognized from it. Any surplus tissue after being used for this study, will be stored in the Liverpool Women's Hospital and may be used for other ethically approved research.

If you are interested in taking part, please contact Dr. Dharani Hapangama (0151- 702 4114 or 0151 706 9988, bleep 141) in the Liverpool Women's Hospital, Crown Street, Liverpool.

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

Appendix V: Patient Consent Form

Liverpool
Women's
Hospital

Study Number:
Patient Identification Number for this trial:

CONSENT FORM

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

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

Please initial box

1. I confirm that I have read and understand the information sheet dated ....
(version ....) for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

3. I understand that sections of any of my medical notes may be looked at by responsible individuals from [University of Liverpool & Liverpool Women's Hospital] or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.

4. I agree to take part in the above study and for my GP to be informed of my part taking.

5. I agree for surplus tissue to be stored in the department of obstetrics & Gyanecology and to be used in other ethically approved studies.

_____   _____   _____
Name of Patient   Date   Signature

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

_____   _____   _____
Researcher   Date   Signature

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Appendix VI: Patient Details Form

Liverpool Women's
NHS Foundation Trust

Study number: LWH0813
Patient Identification Number for this trial:

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

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

Age:

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

Smoking history:

Parity:
Miscarriage:
TOP:

Days of bleeding:
Cycle length:
LMP:

Endometriosis stage:

Appendix VII: Nanodrop Results

Table 21 Nanodrop results for normal endometrial samples

	ng/µl	260/280
N51 +	117.5	1.69
N51 -	939.3	1.89
N54 +	214.1	1.74
N54 -	580.9	1.81
N58 +	64.9	1.66
N58 -	746.1	1.85
N52 +	53.2	1.5
N52 -	715.8	1.82
N53 +	66.7	1.8
N53 -	1089.8	1.94
N55 +	126.8	1.66
N55 -	744.2	1.92
N49 +	124.6	1.7
N49 -	407.0	1.91
N59 +	276.4	1.79
N59 -	758.4	1.85
N77 +	54.5	1.77
N77 -	107.8	1.76
N85 +	7.4	1.62
N85 -	119.3	1.82
N86 +	21.1	1.77
N86 -	62.0	1.90
N95+	31.7	2.45
N95 -	217.6	1.91

Table 22 Nanodrop results for endometriosis endometrial samples

	ng/µl	260/280
E36 +	157.4	1.79
E36 -	602.4	1.81
E40 +	45.3	1.80
E40 -	352.6	1.90
E41 +	249.6	1.77
E41 -	652.5	1.90
E42 +	86.3	1.78
E42 -	412.1	1.91
E43 +	403.0	1.87
E43 -	469.5	1.82
E44 +	6.6	1.68
E44 -	4000.4	1.95
E46 +	618.8	1.90
E46 -	50.2	1.82
E48 +	291.0	1.80
E48 -	736.2	1.90

Table 23 Nanodrop results for organoid samples

	ng/µl	260/280
Pooled N54, N55, N57 +	339.5	1.86
Pooled N54, N55, N57 -	196.2	1.84
Pooled E42, E43, E44 +	778.5	1.89
Pooled E42, E43, E44 -	362.9	1.85

Appendix VIII: Standard Curves

For all qPCR serial dilution experiments: 1x = red, 1/5x= blue, 1/25x= purple, 1/125x= orange, 1/625x=green, 1/3125x= yellow, NTC= pink.

Threshold for all qPCR graphs set at 0.07.

FUT4 Standard Curve
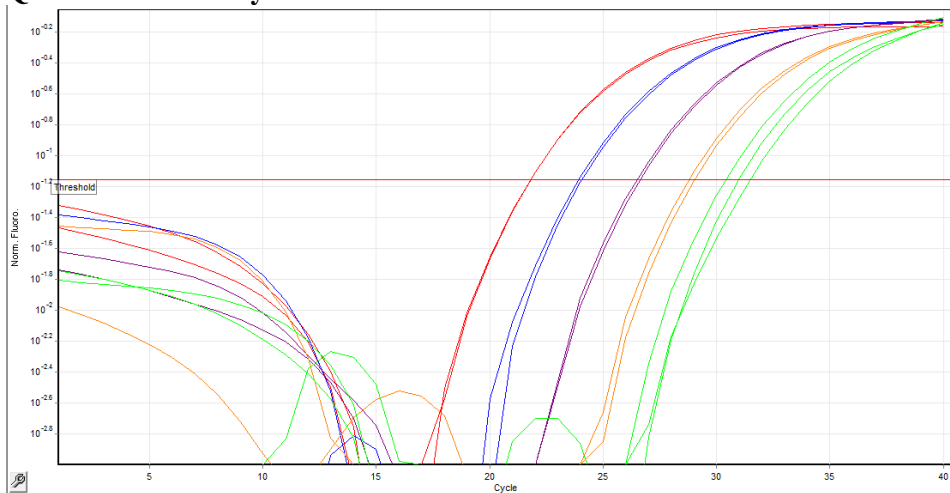
Quantification Analysis:
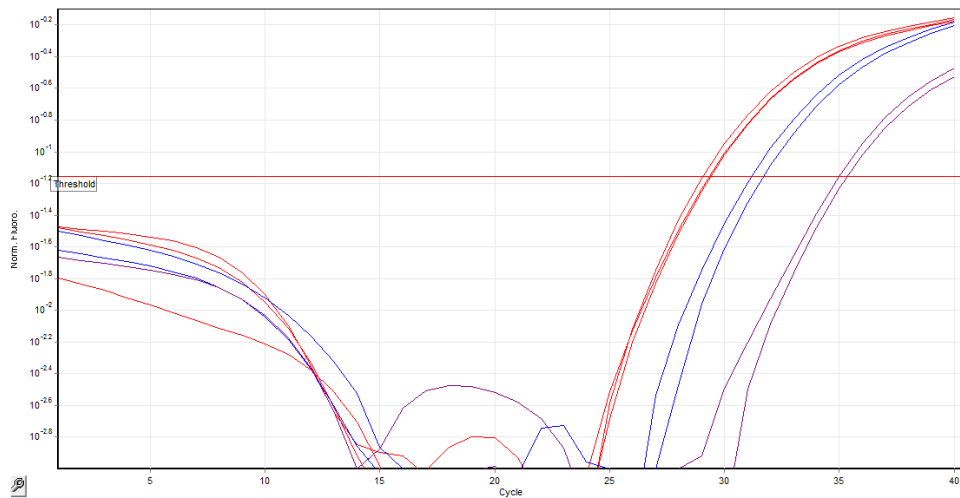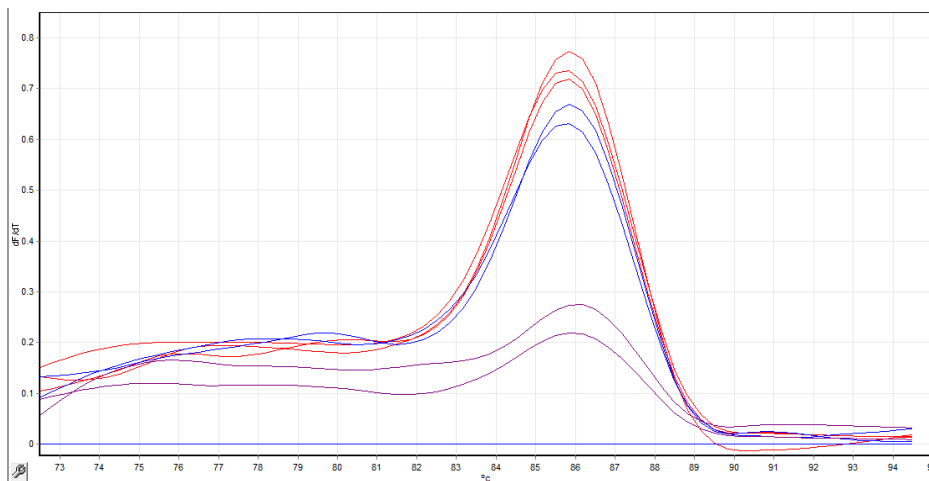
Melt Analysis:

Primer efficiency =0.74
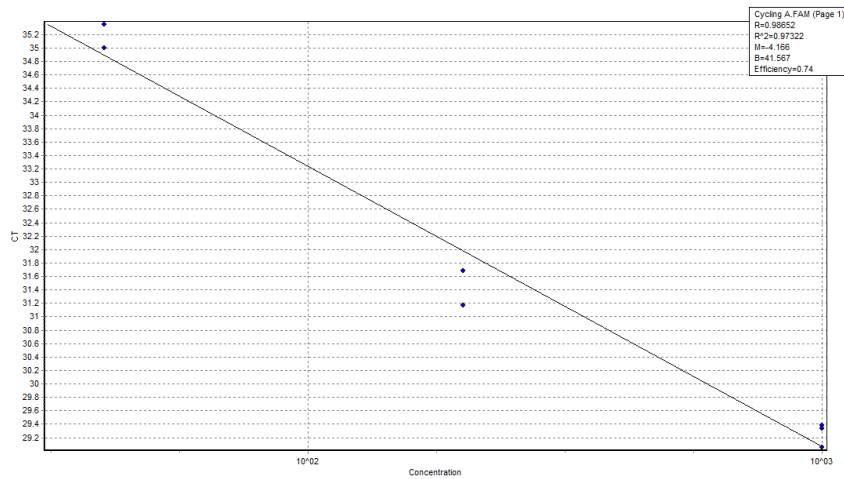
OCT4 Standard Curve
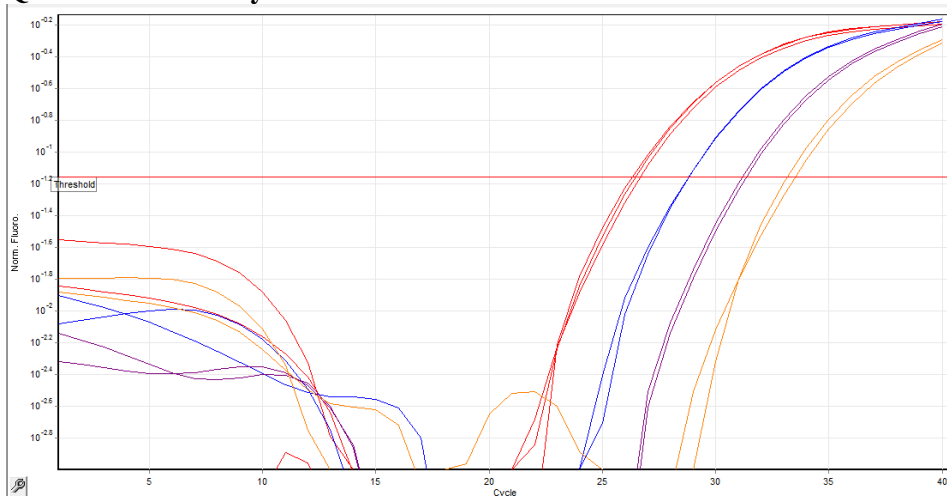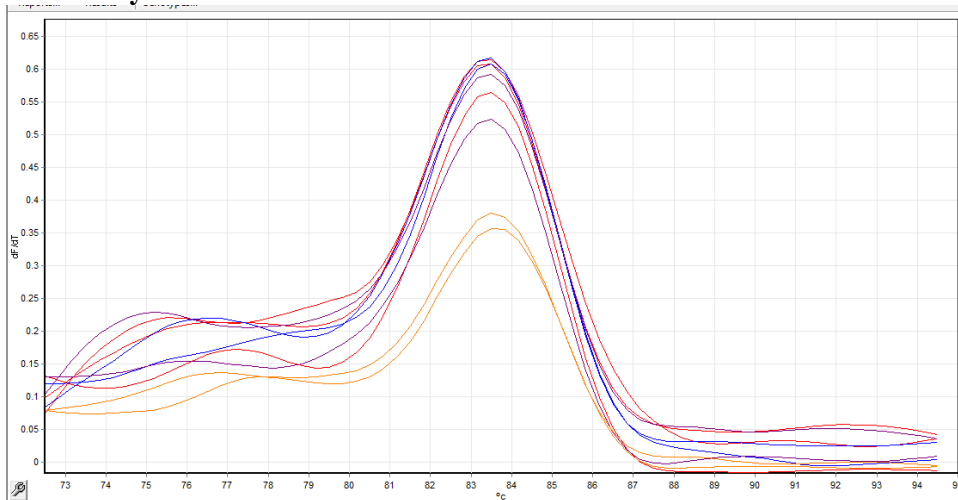
Quantification Analysis:
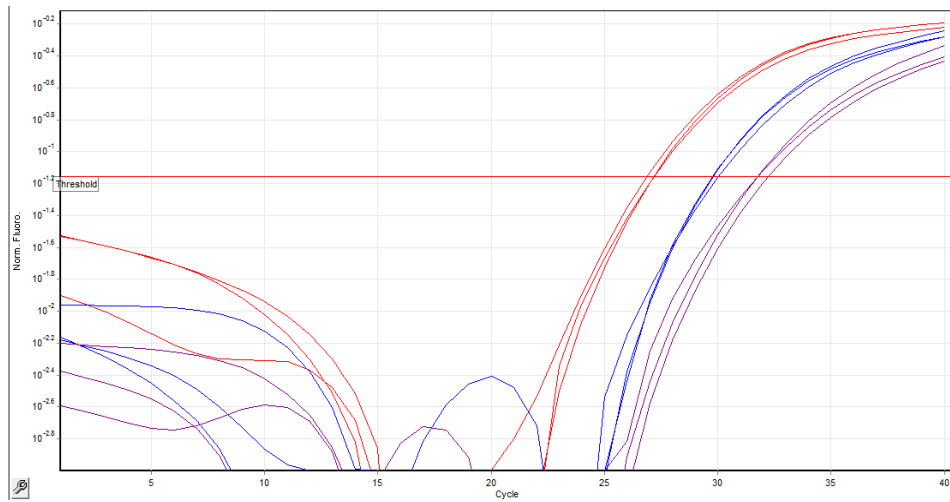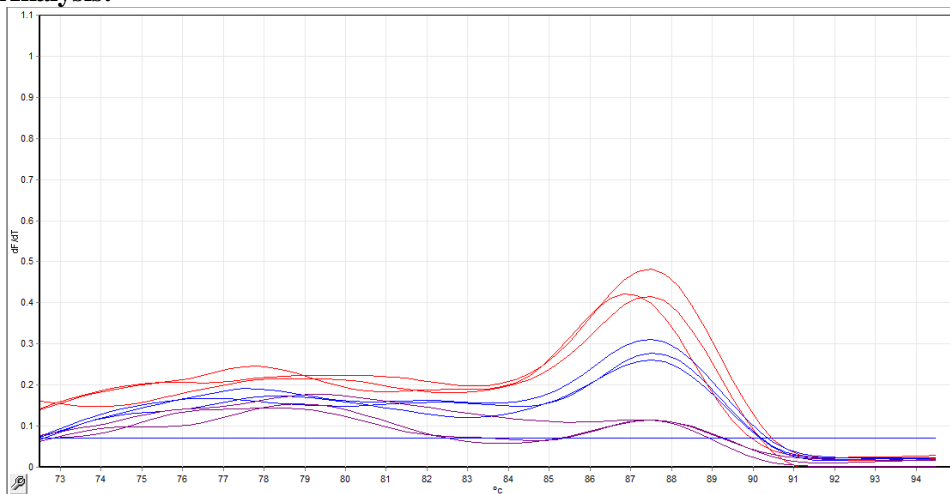
Melt Analysis:

Primer efficiency =0.95
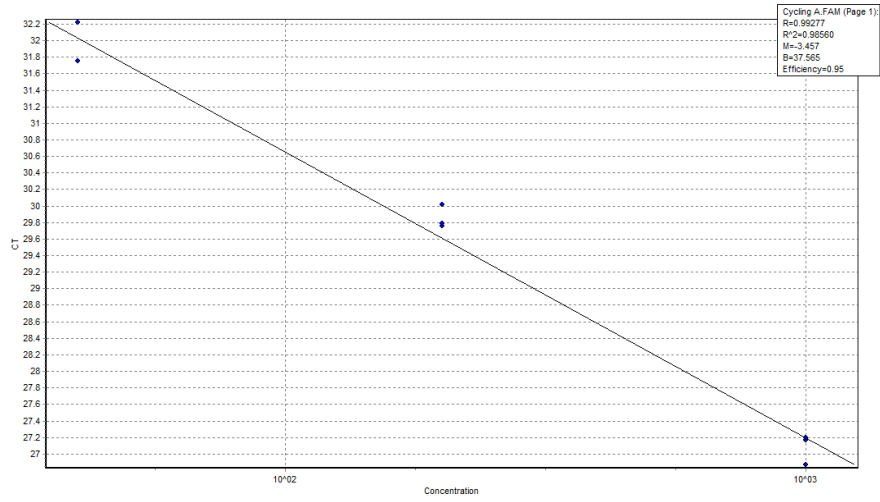
SOX2 Standard Curve

Quantification Analysis:

Melt Analysis:
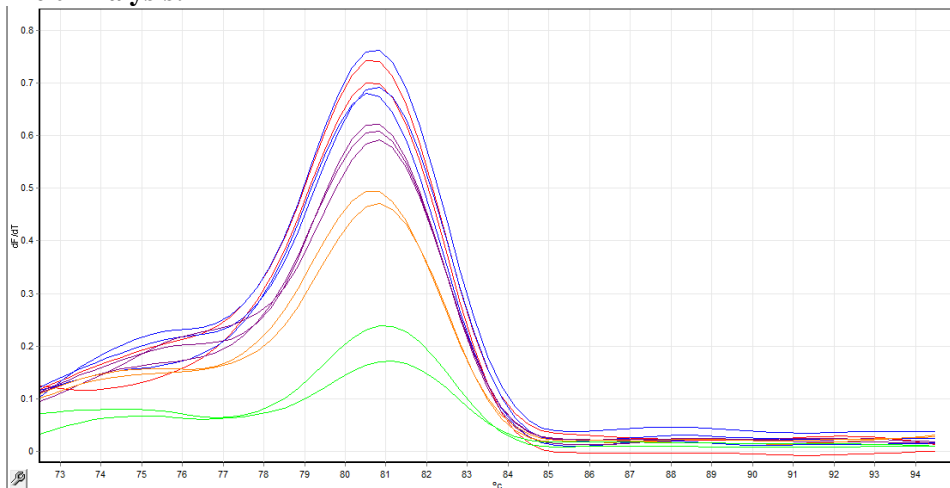

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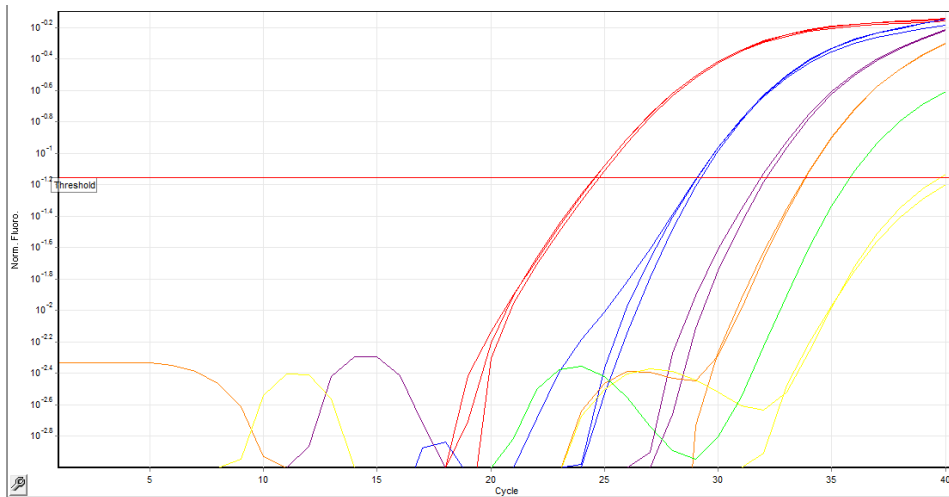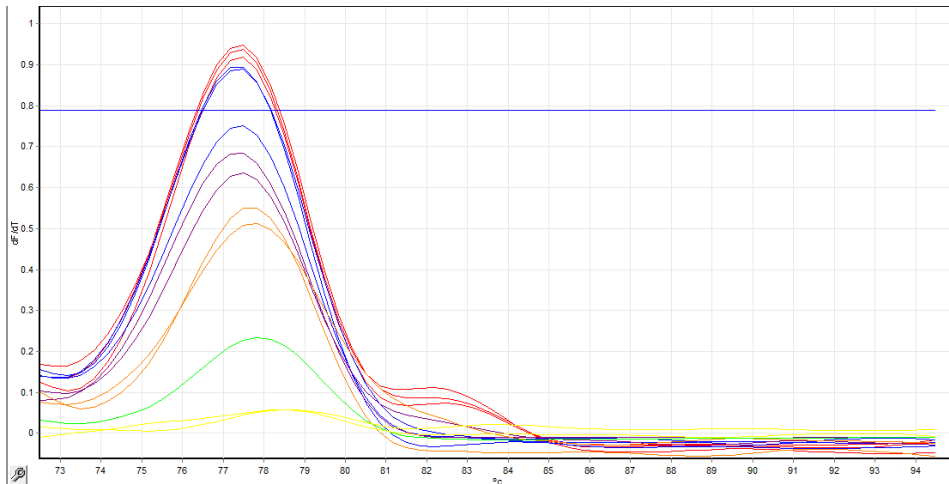
Primer Efficiency= 0.76
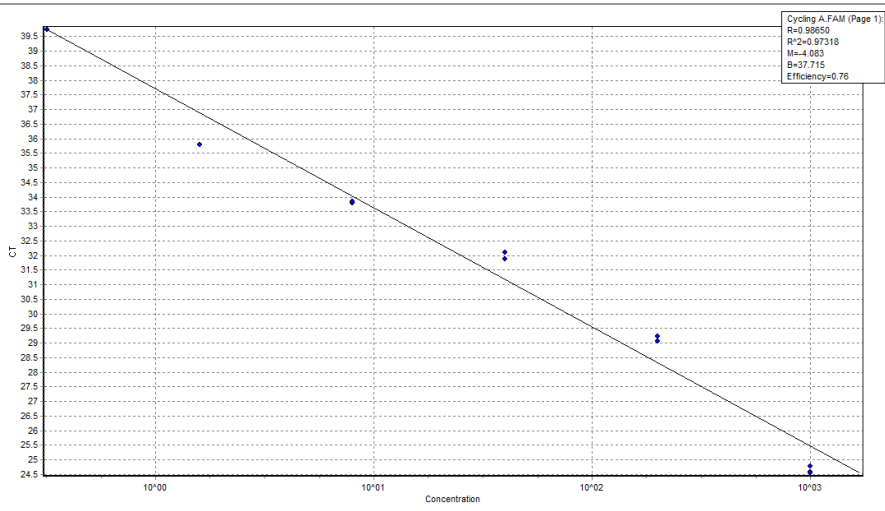
CD9 Standard Curve

Quantification Analysis:
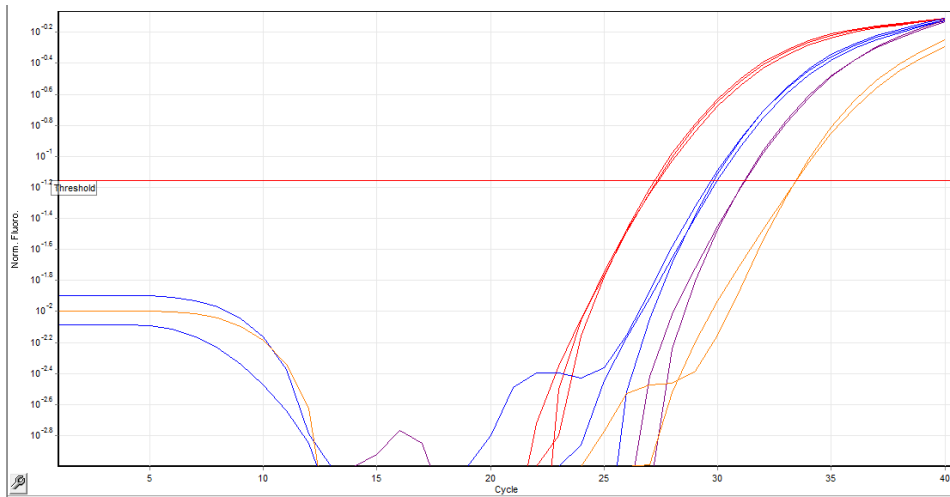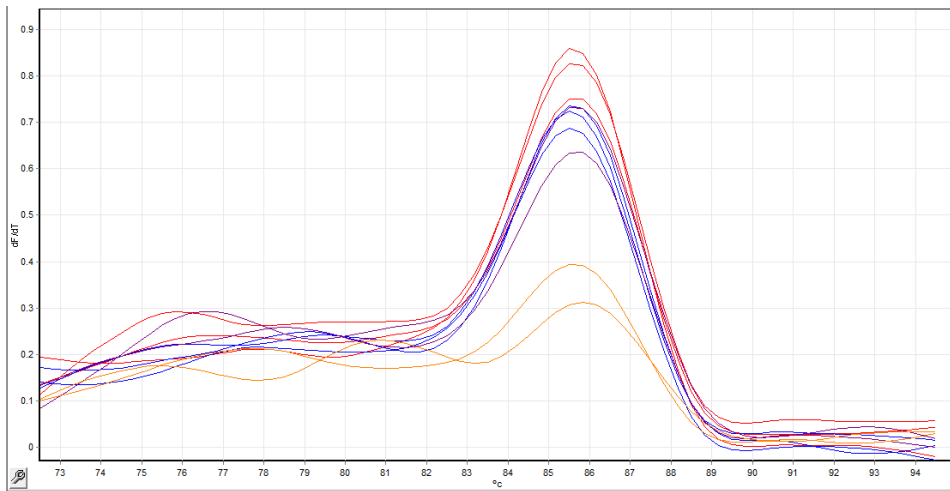
Melt Analysis:

Primer Efficiency= 1.26
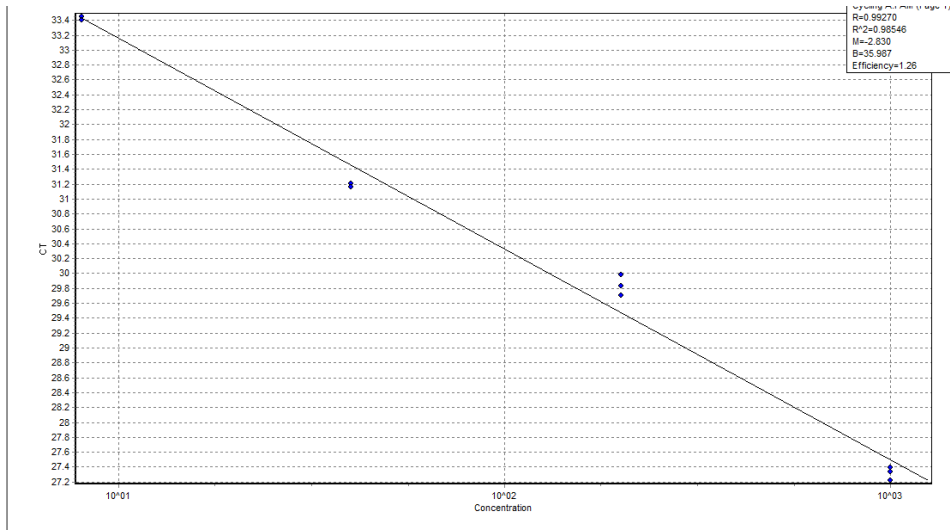
YWHAZ Standard Curve

Quantification Analysis:
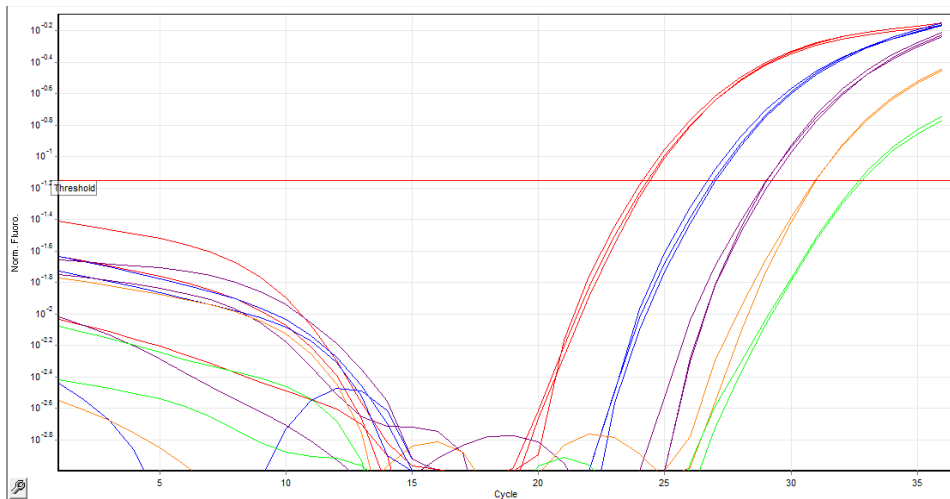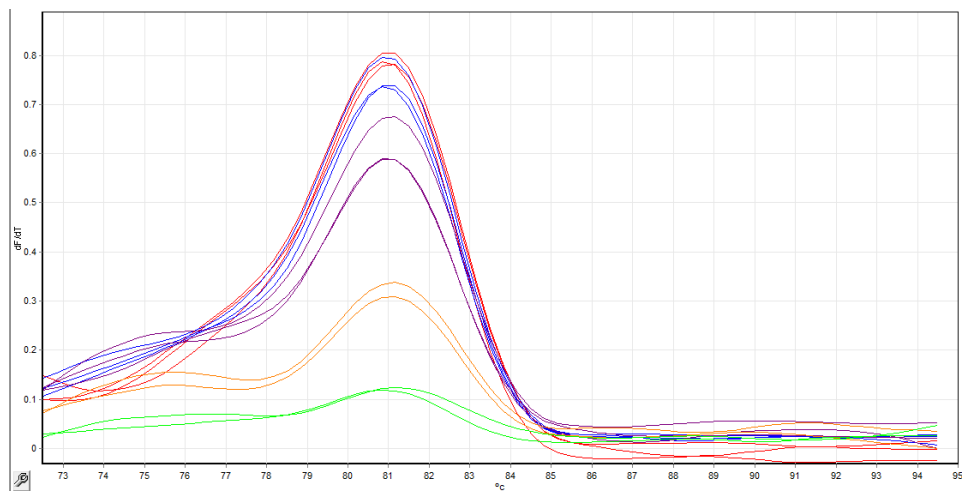
Melt Analysis:

Primer Efficiency= 1.14
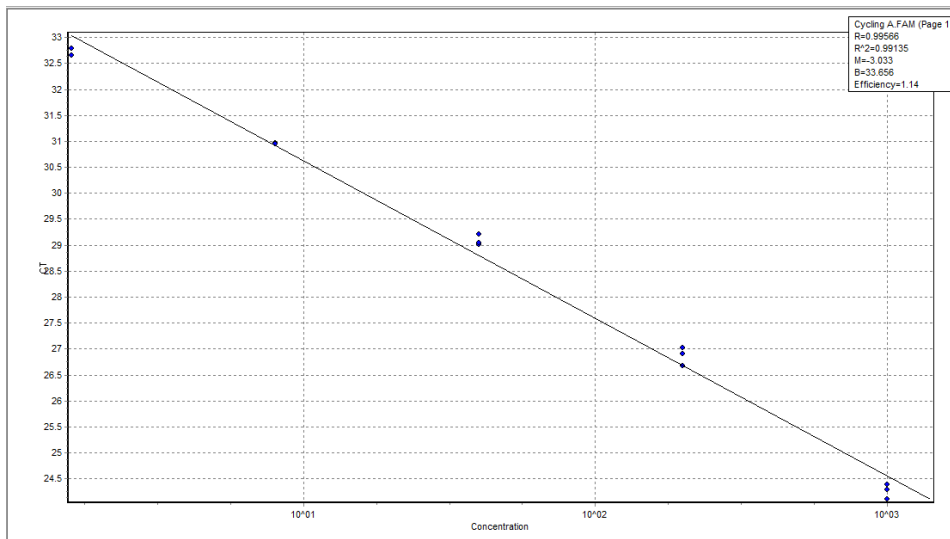
YWHAZ Standard Curve

Quantification Analysis:
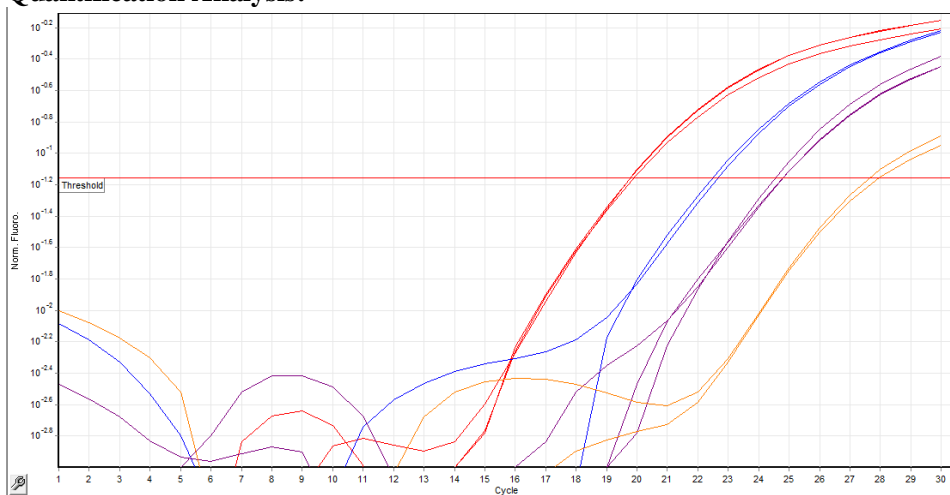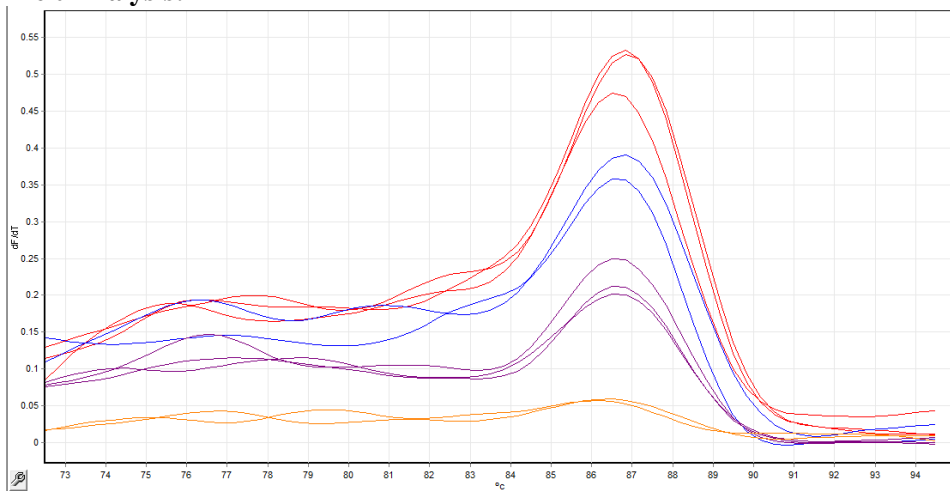
Melt Analysis:

Primer Efficiency= 0.86
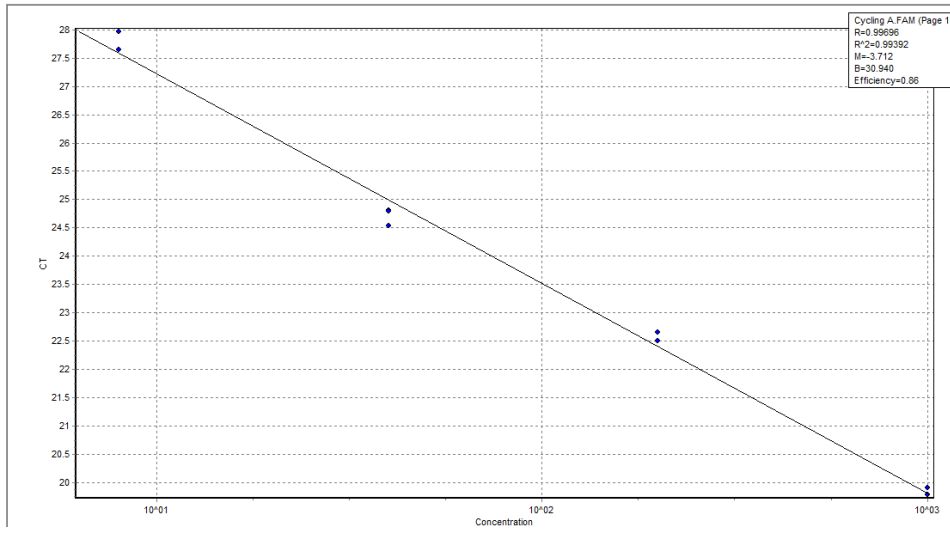
CD133 Standard Curve

Quantification Analysis:
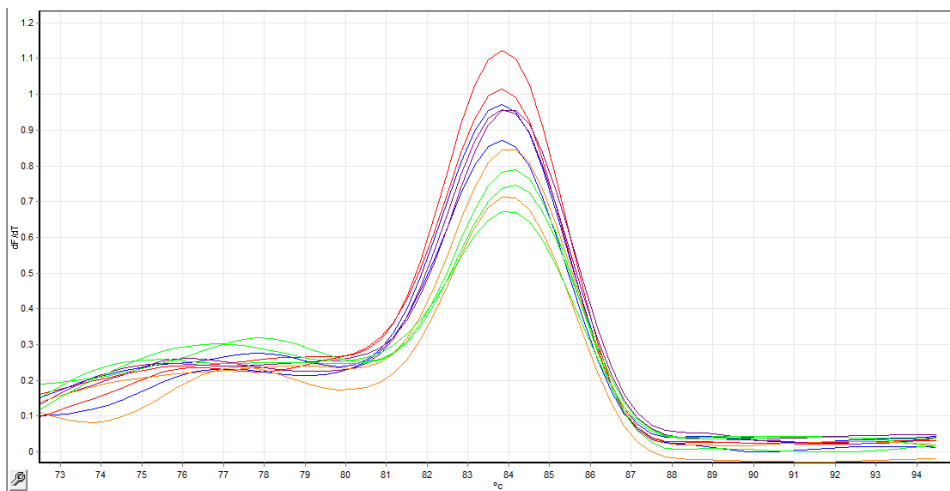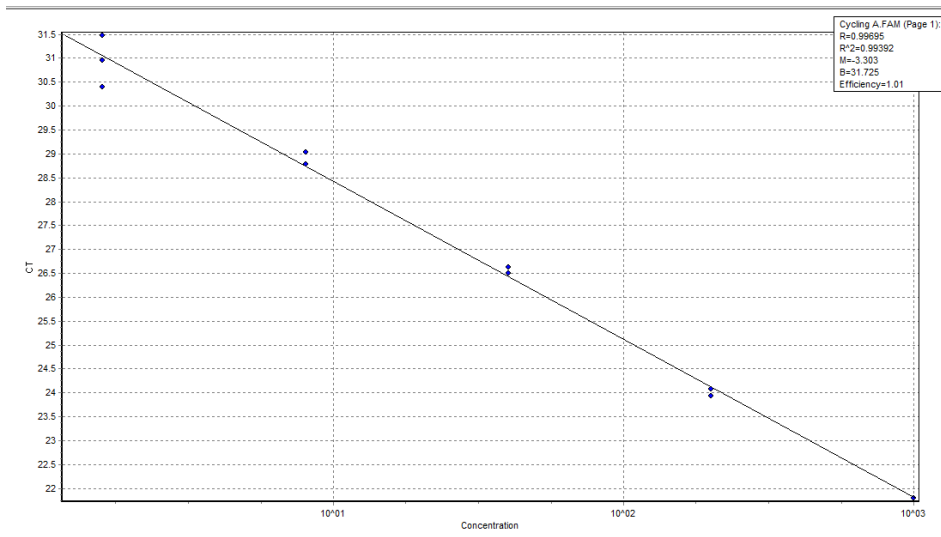
Melt Analysis:

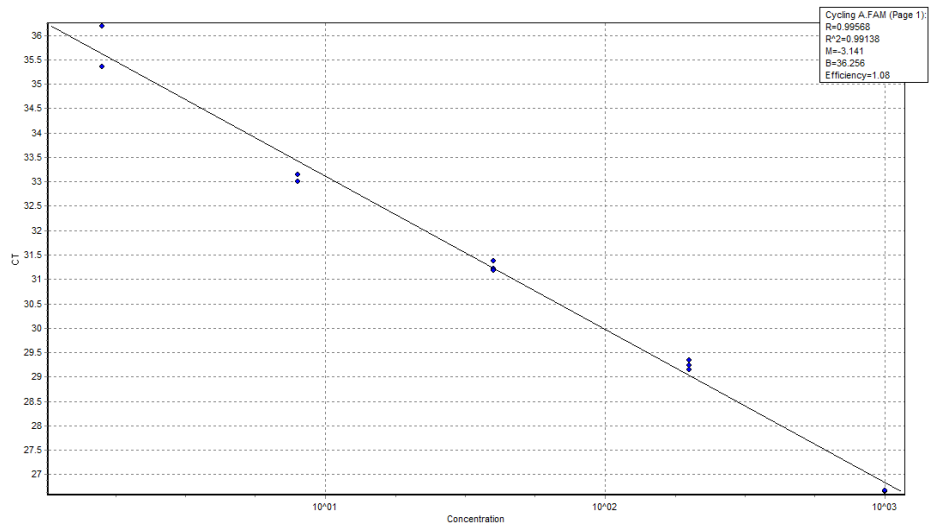**Primer Efficiency= 1.01**

ERα Standard Curve
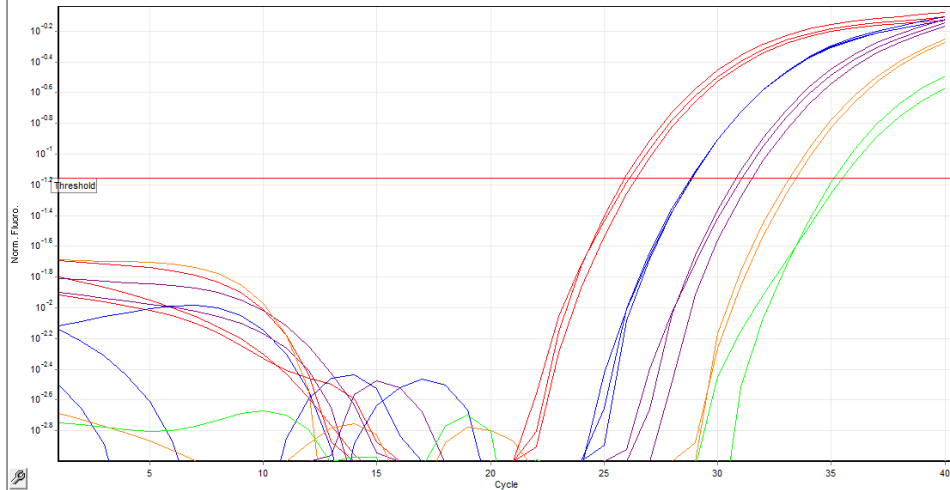
Quantification Analysis:

Melt Analysis:

Primer Efficiency= 1.01
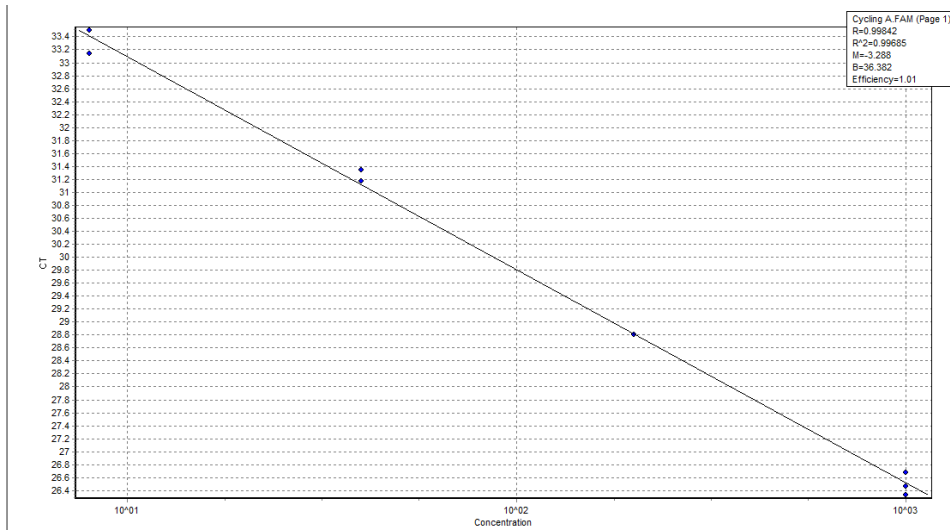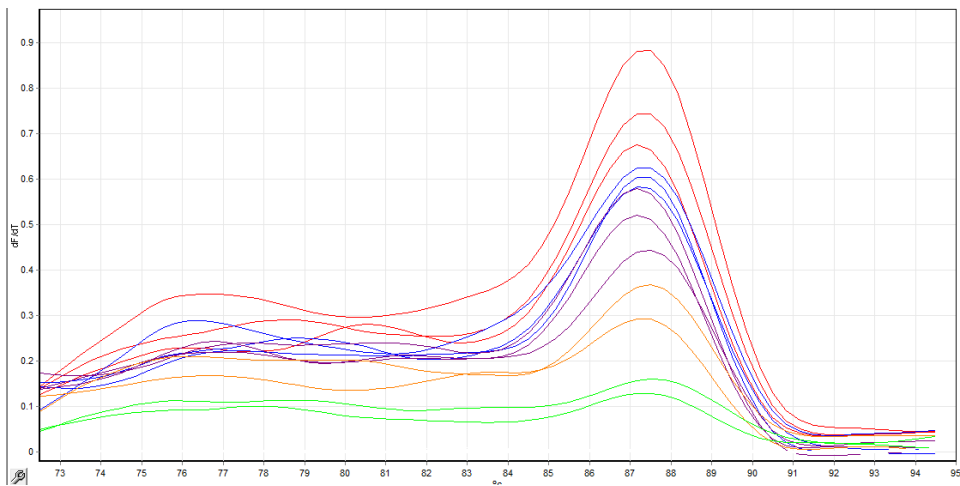

ERα Standard Curve

Quantification Analysis:
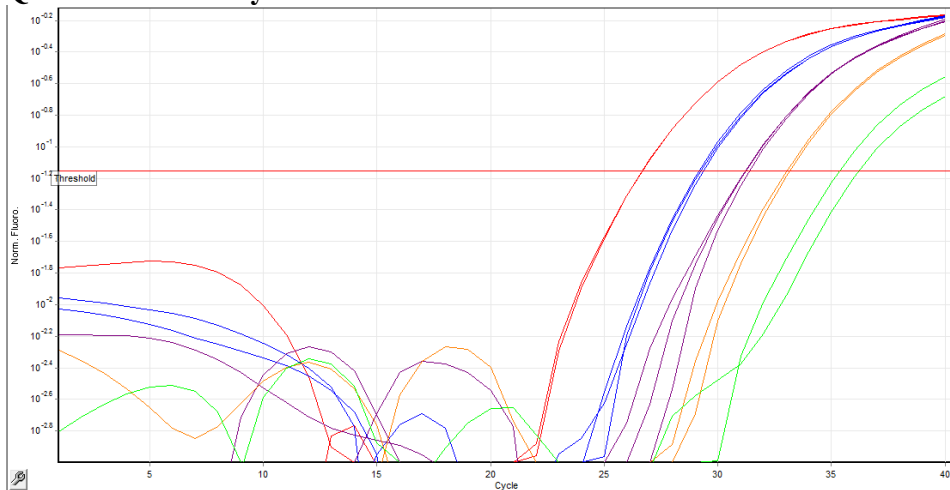
Melt Analysis:

Primer Efficiency= 1.08

ERα Standard Curve

Quantification Analysis:

Melt Analysis:

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Primer Efficiency= 1.02

Cycling A.FAM (Page 1):
R=0.99652
R^2=0.99306
M=-3.276
B=36.197
Efficiency=1.02