Charmaine S.C. Tan

BBiomedSci (Hons)

EPITHELIAL PROGENITOR CELLS IN HUMAN ENDOMETRIUM AND ENDOMETRIOSIS

PhD Thesis

Department of Obstetrics and Gynaecology and

Monash Institute of Medical Research

Monash University

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Contents

Dedication

To my parents, Kim Thai and Jennie.

With love and gratitude.

General Declaration

Monash University

Monash Research Graduate School

Declaration for thesis based or partially based on conjointly published or unpublished work

In accordance with Monash University Doctorate Regulation 17/ Doctor of Philosophy and Master of Philosophy (MPhil) regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes three unpublished manuscripts in preparation for eventual publication. The core theme of the thesis is epithelial progenitor cells in the human endometrium and in

endometriosis. The ideas, development and writing up of all the manuscripts in the thesis were the principal responsibility of myself, the candidate, working within the Ritchie Centre, Monash Institute of Medical Research under the guidance of the main supervisor, Dr. Caroline Gargett.

Signed: Charmaine S.C. Tan

Date: Feburary, 2011

Copyright Notices

Structure of thesis

In compliance with Monash University Doctorate Regulation, this thesis consists of unpublished works relating to "Epithelial progenitor cells in the human endometrium and in endometriosis".

Chapter 1 – *Introduction*. Written as a chapter.

Chapter 2 – *Identifying candidate markers of human endometrial epithelial progenitor cells*. Written as a manuscript in preparation for publication.

Chapter 3 – *HER3 is a putative marker of human endometrial epithelial progenitor cells*. Written as a manuscript in preparation for publication.

Chapter 4 – *Endometrial stem/progenitor cells and endometriosis*. Written as a manuscript in preparation for eventual submission.

Chapter 5 – *Discussion*. Written as a chapter.

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Abstract

The human endometrium is a highly regenerative tissue that undergoes rapid repair and restoration following menstruation and parturition. Endometrial stem/progenitor cells have been hypothesised to be responsible for this. Despite evidence for endometrial epithelial progenitor cells, there are currently no known markers for their prospective identification.

The aim of this thesis was to identify a candidate marker by screening endometrial cells against a panel of known and novel antibodies for immunoreactivity by flow cytometry and immunohistochemistry. H3 was identified as a priority and was assessed for its ability to be a candidate marker. Endometrial epithelial cells were flow cytometry sorted into subpopulations based upon the relative expression of H3 and EpCAM (an epithelial marker). These subpopulations were assessed for stem/progenitor cell properties using functional assays. This thesis demonstrates for the first time that H3 and EpCAM are putative markers that enrich for endometrial epithelial progenitor cells that are clonogenic and undergo self-renewal *in vitro*.

This thesis also investigated the role that endometrial stem/progenitor cells may have in the development of endometriosis. Shedding endometrium and peritoneal fluid were collected from menstruating women with and without endometriosis and assessed for presence of endometrial stem/progenitor cells. Clonal studies did not support the hypothesis that endometriosis is established because of the erroneous shedding of endometrial stem/progenitor cells during menstruation. This was in contrast to preliminary flow cytometry results that detected an increased expression of putative endometrial stem/progenitor cell markers (H3

and W5C5) in samples collected from endometriosis women. Further studies will investigate this. The findings of this thesis have identified a putative marker that will enable further identification of endometrial epithelial progenitor cell markers in future. These markers will allow further characterisation of this rare population and investigations into their potential role in gynaecological disorders such as endometriosis. The current aetiology of endometriosis remains unknown. The identification of endometrial clonogenic cells within endometriotic lesions will provide future examination into what factors in peritoneal fluid may support ectopic endometrial growth so that improvements in treatment can be made.

Abbreviations

ABCG2	ATP-binding cassette transporter G2
ASC	Adult stem cell
Br Ca	Breast carcinoma protein
BrdU	Bromodeoxyuridine
CE	Colony-forming efficiency
CFU	Colony-forming unit
CK8	Cytokeratin 8
CSC	Cancer stem cell
EC	Endometrial cancer
ECC-1	Endometrial carcinoma cell line
eMSC	Endometrial mesenchymal stem cell
EMT	Epithelial-mesenchymal transition
Ep	EpCAM
ER	Estrogen receptor
ESC	Embryonic stem cell
Fz-4/9/10	Frizzled-4/9/10 receptor
H3	HER3
LRC	Label-retaining cells
miRNA	microRNAs
mbMSC	Menstrual blood mesenchymal stem cell-like cells
MSCA-1	Mesenchymal stem cell antigen-1

NOG	NOD/SCID/γ_c^{null}
Pan CK	Pan cytokeratin
PB	Peripheral blood
PCNA	Proliferating cell nuclear antigen
PD	Population doublings
PF	Peritoneal fluid
PMC	Peritoneal mesothelial cells
PR	Progesterone receptor
pRb	Retinoblastoma protein
RT-PCR	Reverse transcription-polymerase chain reaction
SP	Side-population
TA	Transit amplifying
TNF-α	Tumor necrosis factor-α
UMB	Uterine menstrual blood
VMB	Vaginal menstrual blood

CHAPTER 1

Introduction

1.1 Stem cells

The first stem cells were identified 30 years ago in the mouse embryo (Evans and Kaufman, 1981; Martin, 1981). This has lead to a mainstream pursuit of embryonic stem cells (ESC) that has expanded into adult tissues and organs. Earliest adult stem cells (ASC) were studied in the context of the hemapoeitic system resulting in a well characterised stem cell hierarchy and has lead to investigations in other systems such as the brain, skin and mammary gland (Weissman et al., 2001; Morris et al., 2004; Morrison, 2001; Shackleton et al., 2008; Stingl et al., 2001). The understanding of stem cells and their remarkable regenerative properties holds much promise for regenerative medicine. Thus, the unifying aim of these studies are to translate the understandings from the bench to treatment for the bedside.

Stem cells are clonogenic cells, capable of self-renewal and differentiation into a committed cell type (Weissman et al., 2001). During embryogenesis, the inner cell mass of the blastocyst gives rise to the cells of the three germ layers: endoderm, mesoderm and ectoderm (Gadue et al., 2005; Smith, 2006; De Miguel et al., 2010) which subsequently develop to form somatic tissue (Fig 1.1). The innermost layer, the endoderm gives rise to organs of the digestive tract and associated glands, respiratory system and tympanic cavities (Grapin-Botton and

Melton, 2000). The mesodermal middle layer forms the urogenital system, skeletal muscle, the heart, connective tissue, blood cells and the spleen. The external layer, the ectoderm gives rise to the central nervous system, the eye, the epidermis, hair, and mammary glands. ESC are pluripotent, differentiating into all cell types of the three primordial germ layers, including germ cells and some extra embryonic cell types (Smith, 2006). In the absence of inhibitory factors, ESC give rise to teratomas containing somatic cell-derivatives of all three germ layers (Reubinoff et al., 2000; Schuldiner et al., 2000; Reubinoff et al., 2001). In contrast, ASC are multipotent, can persist for a lifetime and function to maintain homeostasis and repair tissue (Fuchs et al., 2004; Diaz-Flores et al., 2006). Since the focus of this thesis is ASC in the endometrium, herein, the term stem cells will refer to adult stem cells, unless stated otherwise.

1.2 Adult stem cells

Adult stem cells can be found in most tissues and organs including the mammary gland, prostate and skin (Hudson et al., 2000; Collins et al., 2001; Stingl et al., 2001; Blanpain et al., 2007; Shackleton et al., 2008). These cells remain quiescent and function to maintain tissue homeostasis by replacing cells lost through natural cellular turnover or in response to injury (Fuchs et al., 2004; Diaz-Flores et al., 2006). Stem cells are difficult to identify being rare (Spradling et al., 2001) and lacking distinguishing surface markers (Ramalho-Santos et al., 2002; Kaur et al., 2004). The best characterised stem cell system is the hematopoietic system where markers are well known for the identification of specific subpopulations of undifferentiated from mature progeny. Furthermore, the hierarchy of somatic cells of the eight lineages emanating from the most primitive hematopoietic is well established. However for most tissues and organs characterisation is in progress and surface markers to differentiate stem cells from their mature progeny are not available. Instead, stem cells have been identified by their functional properties: high proliferative potential, self-renewing capacity and ability

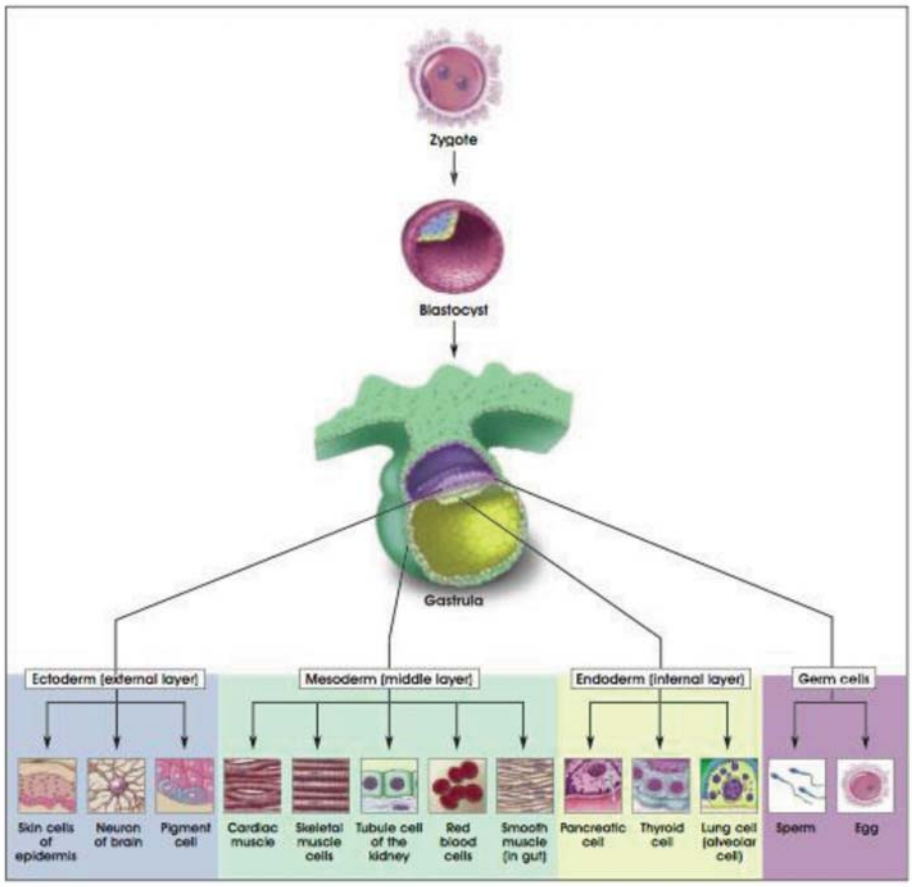


Figure 1.1: Differentiation of human tissues from the three primordial germ layers. Reproduced with permission from ©2001 Terese Winslow (assisted by Caitlin Duckwall)

to differentiate into at least one type of mature functional progeny (Potten and Loeffler, 1990; Morrison et al., 1997; Weissman et al., 2001; Eckfeldt et al., 2005; Gargett, 2007).

1.3 Properties of stem cells

1.3.1 Self-renewal

Self-renewal is the ability to divide and produce identical daughter cells to replenish the life-long stem cell reserve. This division can be symmetrical where two daughter stem cells or two lineage committed daughter cells result, or asymmetrical where one daughter stem cell and

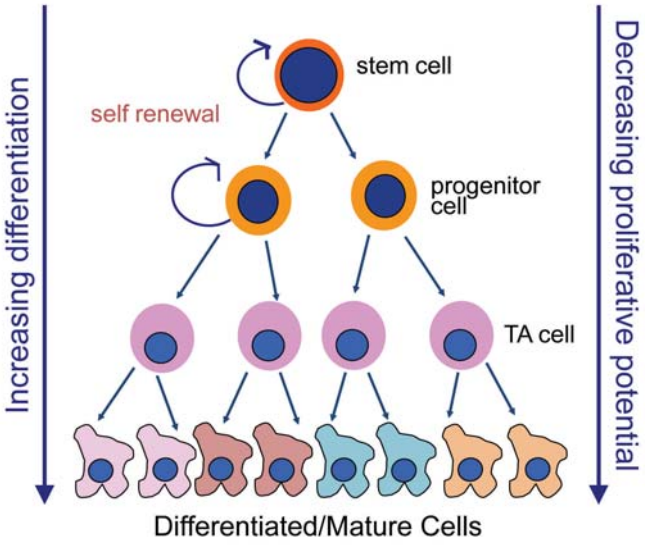


Figure 1.2: Hierarchy of stem cell differentiation. TA, transit amplifying. Adapted with permission from Chan et al. (2004) *Biology of Reproduction* 70(6): 1738-50.

one lineage committed progenitor, a transit-amplifying (TA) cell arises (Bongso and Richards, 2004; Rizvi and Wong, 2005; Diaz-Flores et al., 2006) (Fig 1.2). TA cells do not self-renew but undergo rapid proliferation and are more likely to undergo terminal differentiation into a mature cell type (Jones and Watt, 1993) losing their proliferative capacity (Lajtha, 1979). TA cells are an intermediate between stem cells and mature progeny (Rizvi and Wong, 2005; Diaz-Flores et al., 2006) in the cellular hierarchy (Fig. 1.2) and undergo proliferation to produce the necessary cells required for tissue function (Lajtha, 1979), gradually acquiring differentiation markers as part of this amplification process (Potten and Loeffler, 1990; Jones and Watt, 1993).

1.3.2 Differentiation

Differentiation is when an unspecialised cell becomes a more specialised cell type resulting in changes of genetic expression associated with cellular function rather than division (Potten

and Loeffler, 1990; Morrison et al., 1997; Gargett, 2007). Stem cells display proliferative potentials ranging from the zygote which is totipotent and able to make all embryonic and extra embryonic lineages (Gage, 2000; Eckfeldt et al., 2005) to pluripotent embryonic stem cells (Gage, 2000; Bongso and Richards, 2004) and finally to ASC which have limited differentiation capacity. ASC are frequently multipotent but can also be bi- or unipotent, generally only differentiating into cell lineages of the tissue in which they reside (Diaz-Flores et al., 2006; Smith, 2006; Roobrouck et al., 2008) but not other tissues or a whole organism. The hematopoietic system is an example of multipotency as hematopoietic stem cells are able to undergo self-renewing divisions *in vivo*, differentiating into all mature blood cell lineages to functionally repopulate the ablated hematopoietic system of an irradiated recipient (Roobrouck et al., 2008). Spermatogonial and endothelial stem cells also fulfill the criteria of stem cells however are unipotent thus only differentiate into one mature cell type (Scholer, 2007; Roobrouck et al., 2008). With their limited differentiation capacity, ASC do not form spontaneous tumors upon transplantation (Fuchs et al., 2004), an attractive feature for using them in cell-based therapies.

1.3.3 Proliferative potential

Since stem cells remain undifferentiated, their progeny too would remain relatively undifferentiated compared to TA and differentiated mature cells (Fuchs and Segre, 2000). It is expected that stem cells and their undifferentiated progeny will be able to undergo more rounds of cell division producing greater numbers of mature functional cells than TA cells given their lower position on the stem cell hierarchy (Fig 1.2). The proliferative potential of a cell is calculated by the number of population doublings (PD), that is the number of divisions a cell can undergo to produce a certain number of cells before senescence. To illustrate this, a study of occular epithelia identified three populations of keratinocytes (holoclones, meroclones and paraclones) with different capacities for multiplication (Pellegrini et al., 1999). Following

comparisons of PD, holocones demonstrated the most proliferative potential by generating the greatest number of cell progeny, which together with the formation of the largest colonies suggested that holoclones possessed properties of a stem cell (Pellegrini et al., 1999).

1.3.4 Side-population cells

Studies of hematopoietic stem cells in mice initially reported a differential ability of stem cells to rapidly efflux a DNA binding dye (Hoechst 33342) (Goodell et al., 1997). This small population was readily identifiable as a well separated population of cells in dual wavelength flow cytometry histograms from the bulk population of Hoechst-staining bone marrow cells thus duly named, side-population (SP) cells (Fig 1.3). It was revealed that the expression of ATP-binding cassette transporter G2 (ABCG2) allowed primitive cells to pump out the DNA binding dye (Zhou et al., 2001) while mature cells lack this ATP-binding cassette and are thus stained (Goodell et al., 1996). These SP cells differentiated *in vitro* into cells of all hematopoietic lineages (myelomonocytic, megakaryocytic, erythroid, and T-cell lineage) (Goodell et al., 1996). In a follow-up study, SP cells were reported to reconstitute up to 35% of recipient mice bone marrow (Camargo et al., 2006). From these studies of the hematopoietic system, other stem cells have been successfully identified using the SP method, including the mammary gland, skeletal muscle and skin (Welm et al., 2002; Montanaro et al., 2004). However, the SP phenotype as a definitive stem cell property remains controversial, since one group was unable to demonstrate stem cell properties in SP cells from the epidermis, and non-SP cells had stem cell-like behaviour (Triel et al., 2004). Further, another study found no difference in hematopoietic reconstitution following transplantation whether SP or non-SP cells were used (Morita et al., 2006). However, these discrepancies could be due to technical issues as this methodology is challenging (Montanaro et al., 2004; Tadjali et al., 2006).

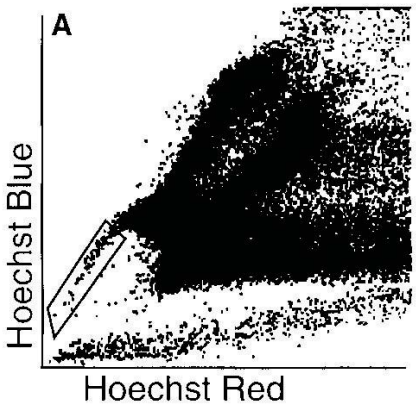


Figure 1.3: Flow cytometry histogram of Hoechst fluorescence of murine bone marrow cells. Side population cells are in the boxed region. Reprinted with permission Goodell et al. (1996) © Rockefeller University Press. *Journal of Experimental Medicine* 183:1797-1806.

1.4 Stem cell niche

Given their life-long existence in the body, stem cells need to be accommodated and protected. Thirty years ago, Schofield coined the term 'niche' in reference to the specialised microenvironment supporting stem cells following investigations into hematopoietic stem cells (Schofield, 1978). The term 'niche' is not merely a physical location but encompasses the cellular and extracellular components of the microenvironment surrounding stem cells. Signals originating from support cells have important roles in regulating stem cell function, especially self-renewal and differentiation cell fate decisions (Li and Xie, 2005). Importantly, niches can indefinitely house one or more stem cell and are able to control their self-renewal and production of progeny *in vivo* (Spradling et al., 2001) as well as control newly introduced exogenous cells to become or to remain as stem cells (Xie and Spradling, 2000). Much of our current understanding of stem cell niches stems from studies of model organisms, the *Drosophila* gonad (Xie and Spradling, 2000) and *Caenorhabditis elegans*, as it has been difficult to isolate stem cells from mammals due to anatomical and structural complexities of most organs (Li and Xie, 2005). Studies of the *Drosophila* have revealed the importance

of cell to cell interactions between germ stem cell and supporting stromal niche cells for maintaining, developing and regulating germ stem cell function (Xie and Spradling, 2000). The niche also functions as an anchor for stem cells with cadherins and adhesion molecules (e.g. integrins) mediating this process (Xie and Spradling, 2000) as well as generating signal molecules to control the fate and number of stem cell as they undergo cell division. These signals are conserved from *Drosophila* to mammals with the Wnt, Bone Morphogenetic Proteins and Transforming Growth Factor-β developmental signaling pathways being three of several important regulatory pathways involved in balancing self-renewal, differentiation and apoptosis (Xie and Spradling, 2000). Despite the conservation of signals between species, each tissue's microenvironment consists of different resident cells and different intrinsic pathways, adding to the complexity in deducing the exact mechanisms involved (Fuchs et al., 2004).

1.5 Assays of stem cell activity

The lack of distinguishing markers has meant an alternative approach be taken to identify and characterise this rare population by relying on assays of stem cell function. These assays confirm and assess the presence of putative stem cell by exploiting the slow-cycling, quiescent nature of stem cells as well as their ability to differentiate into mature progeny. Assays of stem cell activity are: clonogenicity, self-renewal, proliferative potential, differentiation, and *in vivo* tissue reconstitution.

1.5.1 Colony-forming unit activity

Clonogenicity is the ability of a single cell (colony-forming unit; CFU) to initiate a colony of cells when seeded at extremely low densities or by limiting dilution (Gargett and Chan, 2006). This is the simplest method of screening every cell in a population for its ability to undergo substantial cell division (Franken et al., 2006) as the size and morphology of

the colony reflects the heterogeneity of the population and the stem/progenitor cells present. This assay has been extensively used for the initial identification of stem/progenitor cells in characterisation studies of the hematopoietic system (Ash et al., 1981; Lacaud et al., 1998), prostate (Lawson et al., 2007; Miki et al., 2007; Tang et al., 2007), kidney/liver (Dan et al., 2006; Osafune et al., 2006), mammary gland (Dontu et al., 2003) and endometrium (Chan et al., 2004; Schwab et al., 2005).

1.5.2 High proliferative potential

For a tissue to maintain homeostasis, cell production is required which depends upon the self-renewing and proliferative capacity of its stem and lineage-committed TA cells to give rise to mature progeny. The proliferative potential can be calculated as the number of population doublings (PD) in *in vitro* culture that reflect the number of cell divisions required to produce lineage-committed progeny. Comparisons between PD can distinguish between stem cells and their more differentiated counterparts as stem cells have demonstrated significantly greater potential than a TA cell which is lower on the stem cell hierarchy (Pellegrini et al., 1999; Gargett, 2004) (Fig 1.2). For example, to distinguish between the cell types, heterogeneous colonies of mammary epithelial cells were isolated and serially passaged (Stingl et al., 2001). This assay identified a myoepithelial-restricted progenitor that reached a minimum of 16 PD before senescence, demonstrating a significantly higher proliferative potential compared to other cell types identified (Stingl et al., 2001).

1.5.3 Self-renewal

Self-renewal is assessed by serial cloning *in vitro* and serial transplantation *in vivo* which can be combined with limiting dilution to determine the frequency of CFU in a subpopulation of cells (Shackleton et al., 2006). These assays rely upon the ability of the initial CFU to undergo a self-renewing division during colony formation or on transplantation and that the

daughter cell retains the same capacity as her parent cell (Dontu et al., 2003; Dan et al., 2006; Lawson et al., 2007). A study of the mouse mammary gland evaluated the self-renewing ability of a putative mammary progenitor cell using serial transplantation assays into recipient mice. The putative mammary progenitor cell retained full developmental capacity, forming epithelial ductal trees similar to primary mammary glands following serial transplantation three times and thus demonstrated their self-renewal capability (Shackleton et al., 2006).

1.5.4 Differentiation

The *in vitro* differentiation of stem cells is an important hallmark demonstrating a putative stem cell's functional ability to produce mature functional cells of the tissue in which they reside. Putative stem/progenitor cells are usually cultured in bulk or as individual clones before being exposed to differentiation inducing media (Fukuchi et al., 2004) or transplanted. Sufficient time is allowed for cells and tissue explants to differentiate before cells/tissue are stained for tissue-specific markers, histological stains, RNA or protein extracted for gene expression analysis of phenotypic differentiation, functional or tissue-specific markers (Dontu et al., 2003; Gargett and Chan, 2006; Sleeman et al., 2007; Tang et al., 2007). Mammary stem and progenitor cells have been assessed in this approach. Following culture for 16 days in Matrigel (Stingl et al., 2006), a reconstituted gel of basement membrane proteins (Nicosia and Ottinetti, 1990), a proportion of mammary epithelial stem cells produced large structures with a branched ductal appearance and irregular-shaped lumen whilst progenitors produced uniformly spherical acinar structures (Stingl et al., 2006). Gene profiling and immunostaining revealed expression of lineage-specific markers such as keratin 6/8/18/19 (Stingl et al., 2006) providing evidence that purified populations of mammary stem/progenitor cells were capable of differentiation.

1.5.5 Plasticity

Stem cells have been reported to transdifferentiate, whereby an unspecialized cell in one lineage crosses lineage boundaries to differentiate into a cell of an entirely different tissue (Wurmser and Gage, 2002; Wagers and Weissman, 2004; Tarnowski and Sieron, 2006). Studies have reported converting clonally derived neural stem cells into skeletal myotubes (Galli et al., 2000) or into the endothelial cell lineage (Wurmser et al., 2004). The plasticity of bone marrow derived progenitor cells to transdifferentiate into neural cells was also examined (Mezey et al., 2000). To achieve this, transgenic female mice lacking PU.1, a transcription factor exclusively expressed in cells of the hematopoietic lineage, were used as bone marrow recipients for donor male bone marrow. A small population of male derived Y chromosome positive cells were identified in the female central nervous system, co-localising with NeuN, a nuclear protein exclusive to neurons (Mezey et al., 2000), suggesting that bone marrow progenitors had crossed lineages to transdifferentiate into neurons. However, findings from this investigation was disputed as plasticity was only present in a small subset of these neurons. Cell fusion, transplantation of mixed cell populations and presence of an adult stem cell have been proposed as alternative explanations (Wurmser and Gage, 2002; Wagers and Weissman, 2004; Eckfeldt et al., 2005; Lakshmipathy and Verfaillie, 2005). Although transdifferentiation remains a controversial phenomenon it offers increased flexibility of the use of ASC for future cell-based therapies.

1.5.6 *In vivo* tissue reconsitution

Given stem cells function to maintain tissue homeostasis *in vivo* by producing lineage-committed offspring, putative stem cells identified need to also demonstrate this ability *in vivo*. Whilst the aforementioned assays assess stem cell function they are *in vitro*, and thus limited. The *in vivo* reconstitution of tissue is the highest level of assay for demonstrating putative stem cells (Kaur et al., 2004). An isolated stem cell should retain the capacity to

re-populate cells of the tissue from which it was originally isolated. It should also self-renew to protect themselves from damage by replenishing the small pool of tissue reconstituting cells for the maintenance of tissue over time. Putative murine prostate stem cells were assessed for this capability (Lawson et al., 2007). Primary and cultured putative prostate stem cells ($CD45^{-}CD31^{-}Ter119^{-}Sca1^{+}CD49f^{+}$) were injected into the kidney capsule of immunodeficient mice. After 5–8 weeks of transplantation, grafts were harvested and histological staining revealed regeneration of prostatic tubule structures *in vivo* similar to that seen in primary prostatic tissue (Lawson et al., 2007). No tubules were reported for the transplanted depleted fraction of prostate stem cells, indicating that the putative prostate stem cells were capable of reconstituting prostatic tissue *in vivo* (Lawson et al., 2007). Further, the experiments were carried out using differentially genetically marked cells which demonstrated that the regenerated tubules were of clonal origin as chimerism was rarely detected (Lawson et al., 2007), further supporting the identification of prostate stem cells.

1.6 Epithelial stem/progenitor cells

Epithelia are cells that form tightly linked sheets constituting surfaces and linings of the body. They function or form a protective shield against the external environment as well as regulate water, nutrient absorption and glandular secretions (Blanpain et al., 2007). Epithelia can be multilayered (stratified) or single-layered (simple), deriving from the ectoderm, mesoderm or endoderm. In spite of these differences several molecular and cellular characteristics remain similiar (Knust and Bossinger, 2002). During development, epithelia forms from sheets of cells that adhere to basement membrane, rich in extracellular matrix and growth factors (Blanpain et al., 2007) providing structure and supporting the growth of the epithelium and the underlying mesenchyme (Paulsson, 1992; Blanpain et al., 2007). Epithelial cells make cell to cell connections through the formation of desmosomes, adherens and tight junctions that enable intercellular communications for functioning as a sheet (Blanpain et al., 2007). Another

important feature of epithelial cells is their polarized phenotype (apical, basal and lateral) which enables them to withstand mechanical stresses or strong pressures (Knust and Bossinger, 2002). New epithelia are constantly required to replace dead or damaged cells (Blanpain et al., 2007). Stem cells are thought to be responsible for maintaining this homeostasis in epithelial tissue. The homeostatic replacement of cells varies between epithelial tissues. The intestine is well known for its rapid cellular turnover, while others such as the prostate are slower and the mammary gland is cyclical (Blanpain et al., 2007). Many studies have sought to identify these epithelial stem cells for their potential to repair damaged tissues, treat degenerative diseases and examine their purported role in the initiation of cancers.

1.6.1 Intestinal epithelial stem cells

The intestine is a simple columnar epithelium formed in crypt-villus units (Barker et al., 2007) which are continually replaced as cells are shed into the lumen (Bach et al., 2000). Intestinal epithelium also functions to absorb water and nutrients and acts as a protective barrier against ingested pathogens (Blanpain et al., 2007). In the adult, stem cells and their progeny can be found in the base of the crypt region (Barker et al., 2007). Migration out of the crypt region sees the stem cells and TA progeny differentiate into mature cells of four different lineages (enterocytes, goblet cells, neuroendocrine cells and Paneth cells) (Blanpain et al., 2007). Intestinal stem cells were initially identified by characteristics of stem cell function; self-maintenance, proliferation, and differentiation as they lack morphological distingushing features (Vedina et al., 2008; Potten and Loeffler, 1990). Developments in the prospective isolation of murine intestinal epithelial stem cells have identified *LGR5*, a G-protein coupled receptor as a marker. Generation of heterozygous *Lgr5-lacZ* mice enabled the identification of $Lgr5^{+}$ cells at the bottom of intestinal crypts (Barker et al., 2007) (Fig 1.4). Cre-mediated excision of the reported *Lgr5-lacZ* transgenic marker resulted in the irreversible expression of *Lgr5* enabling lineage tracing. Following induction with tamoxifen to activate the Cre

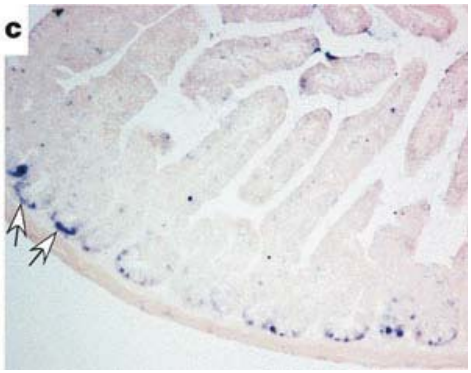


Figure 1.4: Images of *in situ* hybridisation performed on murine intestinal crypts illustrating expression of *Lgr5* at crypt bottoms (white arrows). Reprinted with permission from Barker et al. (2007) *Nature* 449(7165): 1003-7.

reporter, mice were killed at different time points. Over a 60-day period, the $Lgr5^{+}$ cells produced progeny comprising all intestinal epithelial cell types and were responsible for 22–39% of cells emanating from the base of the crypt (Barker et al., 2007). Thus supporting Lgr5 as a marker of intestinal epithelial stem cells. Lgr5 (Barker et al., 2009; Takahashi et al., 2010), and others including CD44, EpCAM (Dalerba et al., 2007) and CD133 (O'Brien et al., 2007; Ricci-Vitiani et al., 2007) have also enabled investigations into the role of stem cells in the malignant colon. These studies have demonstrated that colon cancers contain stem cells providing greater insight into pathogenesis, however further demonstration of their tumorigenic ability awaits.

1.6.2 Prostate epithelial stem cells

The human prostate stores and produces secretions (Huggins et al., 1942) that contribute to semen. The prostate is composed of stratified epithelium consisting of a basal and a secretory layer of columnar cells (Collins et al., 2001; Richardson et al., 2004). In the adult, there is evidence that prostate epithelial stem cells are located in the basal cells of the epithelium (Richardson et al., 2004) and continue to function during androgen withdrawal (Kyprianou and Isaacs, 1988; Tokar et al., 2005; Isaacs, 2008). Due to the lack of surface

markers, prostate stem cells were initally identified through clonal analysis. Two clonal cell types were identified, basal and luminal which were proposed as stem cells and TA cells respectively (Hudson et al., 2000). Adherent basal stem cells demonstrated a 19-fold greater clonogenicity than luminal progenitors and were less likely to express tissue-specific markers (Hudson et al., 2000). Individual colonies of basal cells were harvested and plated into Matrigel, forming spherical structures that contained well organized differentiated epithelium and connecting ducts. These differentiated cells were positive for markers of basal and luminal cells (keratins 5/14/17/19) and androgen receptor indicating multipotency of the basal cells (Hudson et al., 2000). Although these studies support the existence of prostatic stem/progenitor cells, without a marker they cannot be identified *in situ* and differentiated from their progeny. To overcome this, studies have identified putative markers based on known associations of epithelial stem cells with basement membrane molecules such as $\alpha_2\beta_1$-integrin (Collins et al., 2001) or markers used to enrich stem cells in other tissue such as CD133 (Richardson et al., 2004). Human prostate CD133$^+$ cells were identified as a subset (25%) within the $\alpha_2\beta_1$-integrin$^+$ population that demonstrated high clonogenicity and proliferative potential compared to CD133$^-$ and non-adherent basal cells. CD133$^+$ prostate cells took longer to establish colonies perhaps reflecting the slow cycling nature of stem cells (Richardson et al., 2004). CD133$^+$ clonogenic cells, transplanted with human stromal cells produced CD133$^+$ xenografts which demonstrated variable formation of prostate epithelium with expression of prostatic secretions (Richardson et al., 2004), similar to the $\alpha_2\beta_1$-integrin$^+$ stem cells (Collins et al., 2001). These findings will provide a platform for the identification of additional markers to further purify prostate epithelial stem cells, which can lead onto other studies to understand the pathways that govern their regulation and differentiation.

1.6.3 Mammary epithelial stem cells

The mammary gland is a complex tissue that is composed of an epithelial branching network and surrounding stroma (Shackleton et al., 2008). It is generally quiescent however is able to respond to physiological requirements of pregnancy by rapidly proliferating and differentiating to produce the growth and branching of ducts and production of numerous alveoli (Shackleton et al., 2008). Differentiated alveoli epithelial cells secrete milk into the lumen of ducts during lactation. Following lactation, alveoli structures undergo involution returning to their former virgin state ready for the next round of growth and differentiation induced by pregnancy hormones (Blanpain et al., 2007). Mammary stem cells and their TA progeny are thought to line the basement membrane of the terminal bud forming progeny committed to an epithelial or ductal, and during pregnancy a third, myoepithelial cell fate (Blanpain et al., 2007).

Isolation and characterisation of mammary epithelial stem cells have arisen from studies in both human (Stingl et al., 2001; Böcker et al., 2002) and mouse tissue (Stingl et al., 2001; Shackleton et al., 2006). These studies have used a combination of flow cytometry and functional assays *in vitro* (Stingl et al., 2001) and *in vivo* (Shackleton et al., 2008) to characterize the mammary epithelial stem cell population. Three morphologically distinct colonies were observed and cell-specific markers used to identify luminal, myoepithelial and bipotent progenitors. Populations containing only bipotent or myoepithelial-restricted progenitors were assessed for self-renewal using serial passaging with both progenitors demonstrating self-renewal by undergoing three passages in cell culture (Stingl et al., 2001). The use of markers such as EpCAM (epithelial specific antigen), α_6-integrin and MUC1 (plasma membrane glycoprotein) for the prospective isolation of mammary epithelial stem cells produced a 3.5–7 fold enrichment of progenitors in *in vitro* assays (Stingl et al., 2001). Further evidence for self-renewal of putative mammary stem cells was demonstrated by serial transplantation of sorted subpopulations in mice (Kordon and Smith, 1998; Shackleton et al., 2006) using limiting dilution analysis that indicated the frequency of murine mammary

epithelial stem cells to be 1/4,900 cells (Shackleton et al., 2006).

Other markers for the prospective isolation of mammary epithelial stem cells such as CD24, CD29 and CD49f (Shackleton et al., 2006; Stingl et al., 2006) have also been used to enrich for mammary epithelial stem cells which were then confirmed by the reconstitution of a functional mammary gland (Shackleton et al., 2006) in the cleared mammary fat pad assay. Individual sorted $CD45^{-}CD31^{-}TER119^{-}CD29^{hi}CD24^{+}$ cells produced substantial engraftment of the fat pad and histological staining revealed formation of normal ductal structures containing both luminal and myoepithelial cells (Shackleton et al., 2006). Notably, sections derived from pregnant mouse recipients revealed lipid droplets and milk protein within alveoli and ductal lumens respectively, demonstrating high proliferative and multi-lineage capacity of mammary stem cells (Shackleton et al., 2006). The identification of stem cell markers in the normal mammary epithelial gland has validated previous findings and also allowed their use in the investigation of stem cells and their potential cellular transformation in breast cancer (Al-Hajj et al., 2003; Lim et al., 2009).

1.6.4 Isolating epithelial stem cells

Since epithelial stem cells are a rare population and difficult to distinguish from their mature progeny, investigators of a number of epithelial tissues have had to overcome many hurdles successfully prospectively isolate stem/progenitor cells and determine their proliferative and differentiative potential (Stingl et al., 2001; Lawson et al., 2007; McQualter et al., 2010). The first studies initially identified epithelial stem/progenitor cells by dissociating tissue into suspensions of single cells and assessing for colony-forming activity (Stingl et al., 2001). Epithelial CFU were then individually removed from culture to be analyzed by flow cytometry sorting (Stingl et al., 2001). Flow cytometry analysis quantified the percentage of each population (Lawson et al., 2007) and flow cytometry sorted subpopulations were assessed for clonogenic activity (Collins et al., 2001; Stingl et al., 2001; McQualter et al., 2010)

and self-renewal (Shackleton et al., 2006; Lawson et al., 2007; McQualter et al., 2010). *In vitro* and *in vivo* differentiation assays were then used to evaluate the capacity of epithelial stem/progenitor cells to form structures similar to those found in their original tissue (Collins et al., 2001; Lawson et al., 2007; McQualter et al., 2010) or to regenerate their original tissue (Stingl et al., 2006). Transgenic reporter mice were also used as an elegant model that easily identified the origin of mature differentiated progeny (Shackleton et al., 2006). These approaches could be used in the endometrium to identify markers of epithelial progenitors.

1.7 Endometrium

1.7.1 Introduction

The human endometrium is the lining of the uterus that undergoes dynamic changes during the menstrual cycle in preparation for the implanting embryo, resulting in changes in its thickness (Uduwela et al., 2000). In the absence of implantation, shedding of the superficial endometrium occurs in a process known as menstruation. Rapid repair of the surface epithelium ensues and the endometrium regenerates in the next cycle. These processes are all regulated by fluctuating levels of sex steroids in the cycling endometrium (Jabbour et al., 2006; Gargett et al., 2008). The cessation of sex steroidal production sees the endometrium gradually regress to an atrophic state known as menopause (Archer, 2008).

1.7.2 Structure of the endometrium

The uterus is comprised of three layers, the outer serosal covering (perimetrium) and two functional regions, the myometrium and endometrium. The area in which the endometrium and myometrium meet is referred to as the endometrial-myometrial interface and sees no separating submucosal layer between the mucousal endometrium and the smooth muscle myometrium (Uduwela et al., 2000). The endometrium predominantly comprises of epithelial

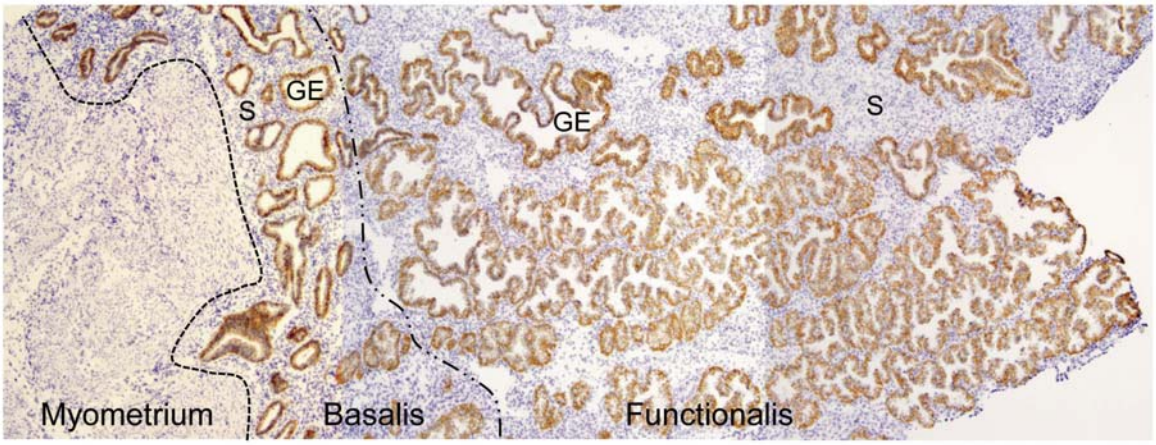


Figure 1.5: Full thickness endometrium stained with epithelial marker EpCAM. The endometrium sits upon the myometrium and is functionally divided into two regions, the upper functionalis and lower basalis. GE, glandular epithelium and S, stroma.

and stromal cells however others including endothelial cells, smooth muscle cells, lymphoid cells, leukocytes and mast cells can also be identified (Chabbert-Buffet et al., 1998). The endometrium consists of two regions; the upper functionalis and lower basalis layer which sits upon the myometrial smooth muscle layer of the uterus (Gray et al., 2001) (Fig 1.5).

The functionalis contains luminal and glandular epithelium loosely held together by stroma. The upper layer is shed during menstruation and regenerates from the remaining basalis (McLennan and Rydell, 1965; Ferenczy, 1976; Gargett, 2007). The basalis is structurally more stable, and consists of terminal ends of branching glands surrounded by dense supportive stroma and scattered lymphoid tissue (Uduwela et al., 2000; Spencer et al., 2005). The uterine and ovarian arteries pass through the myometrium forming the arcuate arteries which after passing through the endometrial-myometrial interface, branch into the anastomosing arteries to supply the basalis and spiral arterioles to supply the functionalis (Dockery, 2002).

1.7.3 Development of endometrial glands

In humans, uterine adenogenesis, the process of endometrial gland development begins at gestational day 140. Adenogenesis involves differentiation of the primordial glandular epithelium buds into luminal epithelium. Tubular glands emanate from the buds, growing in a radial pattern penetrating through the endometrial stroma towards the myometrium (Spencer et al., 2005). At birth, the neonatal human uterus resembles that of the adult, but less developed (Gray et al., 2001). By age six, endometrial glands have extended half-way to the myometrium. At puberty endometrial glands have finished their extensive coiling and branching morphogenesis and are histologically and functionally mature (Gray et al., 2001; Spencer et al., 2005).

1.7.4 Menstrual cycle

The length of a normal menstrual cycle varies between each individual but is conventionally described as 28 days. It can be described in three phases: proliferative (day 5–14), secretory (day 14–28) and menstrual (day 1–4) (Dockery, 2002; Jabbour et al., 2006).

1.7.4.1 Proliferative

The proliferative phase follows menstruation and is controlled by estrogen released from the ovary during follicular growth. This phase is characterised by re-epithelialisation which occurs in the absence of estrogen (Kaitu'u-Lino et al., 2007) and growth of glandular and stromal constituents which are under the influence of increasing estrogen levels (Fig 1.6). During the early proliferative phase (day 5–7) glands elongate and appear as straight and undifferentiated cells with a circular cross-section in histological sections (Dockery, 2002). Glands are lined by ciliated and non-ciliated columnar epithelium with basally located nuclei (Colville, 1968; Ferenczy, 1976; Ludwig and Spornitz, 1991). By mid-proliferative phase (day 8–10) glands have elongated and are slightly tortuous. Glandular cells appear pseudostratified

with mitotic figures. During late proliferative (day 11–14) glands are markedly tortuous with wider lumena and the cells are tall and columnar (Dockery, 2002).

1.7.4.2 Secretory

The secretory phase follows ovulation, during which high levels of progesterone and estrogen are released by the ovarian corpus luteum (Fig. 1.6). During the early secretory phase, high levels of progesterone cause rapid and dramatic changes in the endometrium. Initially the epithelium is pseudostratified and their lumina is partially obliterated as secretory material is generally absent (Dockery, 2002). By mid-secretory phase, glycogen-rich secretions fill the epithelial glandular lumina resulting in maximal gland cell volume and push nuclei to the centre of the cell (Dockery, 2002). Undifferentiated glandular epithelium has differentiated into taller cells and are less pseudostratified. By late secretory, the glands have become even more tortuous with the epithelium characterized by dilated tufts creating a serrated appearance due to the copious release of secretory products (Novak and Te Linde, 1924; Dockery, 2002; Spencer et al., 2005).

1.7.4.3 Menstruation and regeneration

Menstruation occurs in the absence of pregnancy and is the induction of uterine bleeding by the withdrawal of progesterone (Smith, 2002; Salamonsen, 2003) from an estrogen primed endometrium (Jabbour et al., 2006). The function of this extensive tissue remodeling is unclear, however is probably related to its preparation for implantation (Smith, 2002). The endometrium retains a unique capacity to re-epithelialize in the absence of scarring (Ludwig and Spornitz, 1991). Repair begins even while breakdown is underway in adjacent areas (McLennan and Rydell, 1965; Kaitu'u-Lino et al., 2007; Garry et al., 2009), a rapid process that by the fourth day, two-thirds of the entire superficial endometrium has been newly

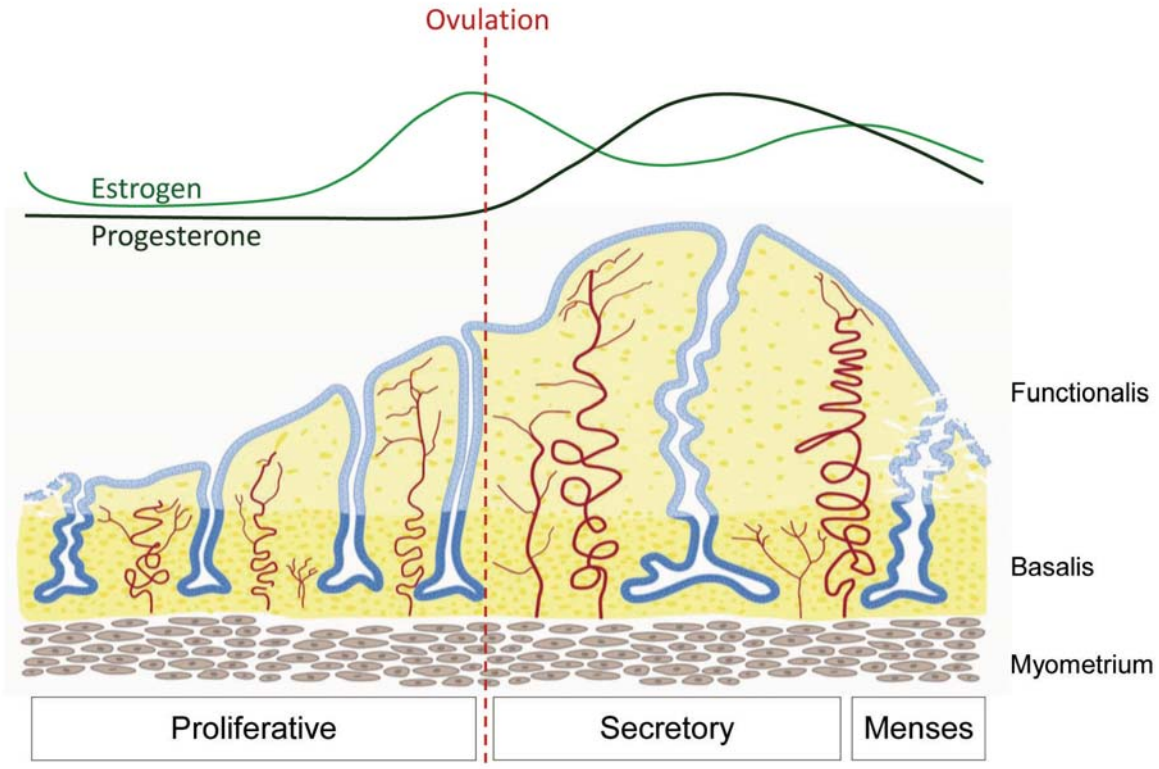


Figure 1.6: Schematic diagram of the menstrual cycle illustrating the changes in the endometrium and regulatory sex steroidal hormones (estrogen and progesterone). Adapted from © Gargett et al. (2008), *Molecular and Cellular Endocrinology*, 1-2: 22-9, with permission from Elsevier.

formed (Ludwig and Spornitz, 1991). Interestingly, not all areas of superficial endometrium are shed (Novak and Te Linde, 1924; McLennan and Rydell, 1965; Garry et al., 2009) and instead remain attached to become incorporated in the new area of proliferation (McLennan and Rydell, 1965). Widely accepted theory postulates that glands regenerate from those remaining in the basalis (Novak and Te Linde, 1924; Ferenczy, 1976; Kaitu'u-Lino et al., 2010). However despite the rapid regeneration of the epithelium, the absence of mitosis in the basalis is difficult to explain (Novak and Te Linde, 1924). One school of thought hypothesises neighbouring adjacent stromal cells are responsible for this regeneration (Garry et al., 2009) as residual glandular epithelium was reported to be metabolically inactive during

menstruation (Baggish et al., 1967; Garry et al., 2010). However further evidence of stromal transdifferentiation is needed.

1.7.4.4 Steroidal hormone regulation of menstruation

The endometrium is a target of the female sex hormones and their importance is illustrated in post-menopausal women, who following treatment with exogenous hormones have regenerated their endometrium and have had successful pregnancies (Paulson et al., 2002). The proliferative and secretory phases are primarily controlled by ovarian produced estrogen and progesterone (Mylonas et al., 2004; Jabbour et al., 2006; Gargett et al., 2008) (Fig. 1.6) and act via estrogen receptors (ER) and progesterone receptors (PR) expressed on endometrial epithelial and stromal cells. Estrogen is produced during ovarian follicular phase and initiates the proliferation of the endometrial epithelium (Mylonas et al., 2004; Jabbour et al., 2006). Two types of ERs exist; alpha (ERα) and beta (ERβ). ERα is the dominant receptor in the adult endometrium (Koehler et al., 2005) with greater immunostaining intensity on glandular epithelium compared to expression of ERβ which remains relatively low throughout the menstrual cycle (Leece et al., 2001). Similarily, two types of PRs exist, A (PR-A) and B (PR-B) and an increase in estrogen promotes the expression of the former (Bethea and Widmann, 1998). Studies using progesterone receptor knock-out mice revealed actions of PR-A are anti-estrogenic, thus inhibit endometrial growth and induce differentiation of glandular and stromal cells (Conneely et al., 2001; Jabbour et al., 2006; Gargett et al., 2008). Conversely, PR-B enhances epithelial proliferation in response to estrogen alone and to both estrogen and progesterone. The concentration of steroidal receptors fluctuates in response to circulating levels of steroidal hormones (Moutsatsou and Sekeris, 2006), indicating the complexity of steroid hormones and receptor interactions for the regulation of cyclic growth and differentiation of the endometrium.

1.8 Endometrial stem/progenitor cells

The human endometrium is a highly regenerative tissue rapidly repairing and growing 4–6 mm in thickness following menstruation (McLennan and Rydell, 1965) in approximately 7–10 days (Gargett, 2007), with this cycle repeating every 28 days from puberty to menopause. Endometrial stem/progenitor cells have been proposed as being responsible for this capacity (Prianishnikov, 1978; Padykula, 1991; Gargett, 2007). The focus of this thesis is on human endometrial epithelial progenitors and this section will therefore predominantly review current understanding of this cellular population rather than endometrial mesenchymal/stromal stem cells.

1.8.1 Indirect evidence

The remarkable regenerative capacity of the endometrium is best demonstrated by clinical observations. Following extensive curettage, parturition and ablation, the endometrium is still able to regenerate and support pregnancy (Wood and Rogers, 1993; Tresserra et al., 1999; Abbott and Garry, 2002; Gargett, 2007). Histological studies of the endometrium demonstrated that post-menopausal women placed on hormone replacement therapy showed proliferative, secretory or inactive patterns (Feeley and Wells, 2001). Further study reported the successful conception and delivery of term pregnancies in women over 50 years of age (Paulson et al., 2002). These observations support the presence of stem cells in the human endometrium. Further, clonality of endometrial epithelial cells was examined by PCR amplification of the androgen receptor gene and results indicated that the cellular composition of the glands was monoclonal (Tanaka et al., 2003). This observation extended to the surrounding glands which also shared clonality, suggesting the presence of more primitive cells that develop into a stem cell population within each gland during development (Tanaka et al., 2003). Counting of epigenetic errors and mathematical modeling have also indicated that each gland consists of a stem cell niche with multiple long-lived stem cells rather than

a single stem cell (Kim et al., 2005). Methylation patterns of endometrial glands revealed an age-related increase in methylation and relatively constant gland diversity indicating the life-long persistence of stem cells in the endometrium (Kim et al., 2005).

1.8.2 Direct evidence

1.8.2.1 *In vitro* functional assays

Cloning studies of human endometrial cells have demonstrated the existence of two separate stem/progenitor cell populations, epithelial and stromal (Chan et al., 2004).

These populations formed two distinct colony types (Chan et al., 2004). The small loosely packed colonies were proposed to be initiated by TA cells and the large dense colonies initiated by stem cells as they had higher proliferative potential and underwent self-renewal (Chan et al., 2004; Gargett et al., 2009). Importantly, clonogenic epithelial cells were isolated from the inactive endometrium albeit with reduced activity (Schwab et al., 2005). Nevertheless, the data supports the life-long persistence of epithelial stem/progenitor cells.

In the epithelial fraction, 0.22% of cells were clonogenic despite large variation between patient samples (Chan et al., 2004) and between proliferative and secretory stages of the cycle (Schwab et al., 2005). Cloning efficiency of large colonies was constant during proliferative ($0.07 \pm 0.04\%$), secretory ($0.07 \pm 0.05\%$) and notably in the inactive ($0.06 \pm 0.05\%$) endometrium (Schwab et al., 2005). Limiting dilution assays of freshly isolated $EpCAM^+$ epithelial cells demonstrated a 1/174 frequency of clonogenic epithelial cells (Gargett et al., 2009). These clonogenic cells underwent substantial self-renewal and were able to be serially cloned greater than three rounds and survived more than four months in culture (Gargett et al., 2009). Observations of large epithelial CFU indicated more differentiated progeny with decreasing proliferative potential at each subsequent subcloning (Gargett et al., 2009), similar to observations in the epidermis (Barrandon and Green, 1987). Large epithelial CFU underwent approximately 40 cell generations (Gargett et al., 2009), two-fold less than epidermal CFU

(Barrandon and Green, 1987) reflecting differences in epidermal and endometrial cellular turnover (Gargett et al., 2009). Clonally derived and expanded epithelial cells were able to form cytokeratin+ gland-like structures, suggesting that endometrial epithelial CFU have the unilineage capacity to differentiate into mature glands *in vitro* (Gargett et al., 2009).

1.8.2.2 Studies on mouse endometrial epithelial progenitor cells

Animal models offer experimental opportunities incompatible with the ethical boundaries of working with humans. Using this to an advantage, candidate epithelial progenitor cells have been identified as label-retaining cells (LRC) in the mouse endometrium (Chan and Gargett, 2006; Kaitu'u-Lino et al., 2010). The LRC technique involves the labelling of cells with a DNA synthesis dye such as bromodeoxyuridine (BrdU) (Chan and Gargett, 2006; Cervello et al., 2007; Szotek et al., 2007; Kaitu'u-Lino et al., 2010). Slow proliferating stem/progenitor cells retain the nucleotide dye following prolonged chase periods, compared to rapidly proliferating TA cells in which the dye rapidly dilutes to undetectable levels in subsequent cell divisions (Chan and Gargett, 2006; Cervello et al., 2007; Szotek et al., 2007; Kaitu'u-Lino et al., 2010). A small population (3%) of endometrial epithelial LRC ($BrdU^+$) cells were reported in adult mouse (Chan and Gargett, 2006).

Extending these findings, a mouse model of endometrial breakdown and repair revealed glandular LRCs (25%) present in the basal area adjacent to the myometrium (Kaitu'u-Lino et al., 2010). Prior to breakdown, a majority ($0.81 \pm 0.39\%$) of glandular epithelial cells were not proliferating (proliferating cell nuclear antigen, $PCNA^-$) however a significant increase in proliferation ($32.2 \pm 9.9\%$) was observed immediately following repair (Kaitu'u-Lino et al., 2010) (Fig. 1.7), indicating their role in maintenance of epithelial tissue homeostasis. Importantly, these glandular epithelial cells retained the BrdU label longer than luminal cells suggesting that the progenitor cell population reside within the glandular epithelia whereas luminal epithelial LRC could represent TA cells (Kaitu'u-Lino et al., 2010). Despite the biological differences between human and mouse, findings from LRC studies of the mouse

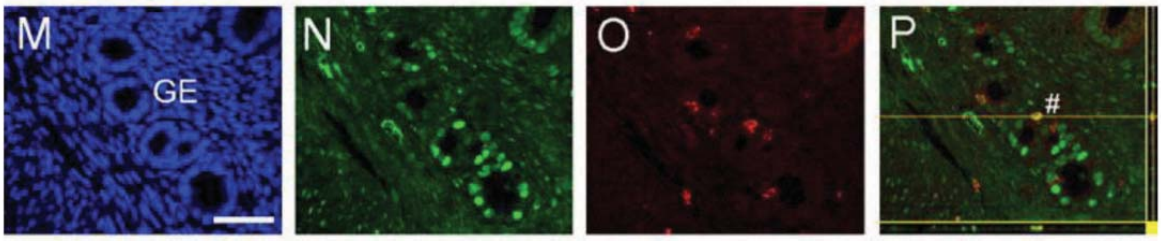


Figure 1.7: Endometrial epithelial label-retaining cells in a mouse model of endometrial breakdown and repair. Immunofluorescence during repair demonstrates (M) DAPI nuclear staining, localisation of (N) PCNA proliferative marker and (O) BrdU in GE, glandular epithelium. #, a double-stained glandular epithelial LRC as shown by (P) optical section. Bar: 50 μm. Reproduced with permission from Kaitu'u-Lino et al. (2010) *Endocrinology* 151: 3386-95.

endometrium strongly support the presence of slow-cycling epithelial progenitor cells in the human endometrium.

1.8.2.3 Expression of stem cell genes

The expression of stemness-related genes in the human endometrium has been investigated. NAC1, a transcription factor involved in self-renewal and maintenance of pluripotency was detected at significantly higher levels during the early- and mid-proliferative stages than other stages of the menstrual cycle (Ishikawa et al., 2010). Similarly, Musashi-1 a marker of intestinal stem cells was detected by quantitativePCR in the endometrium (Götte et al., 2008). In proliferative endometrium, immunohistochemistry was used to demonstrate significantly greater numbers of Mushashi-1-positive cells in the stroma and glands of the basalis than the functionalis suggesting that more stem cells are present in the former (Götte et al., 2008). Additional quantitative-PCR analysis revealed expression of *KLF4*, *BMI1* and *OCT4* genes in all human endometrial (n=14) samples examined (Forte et al., 2009).

Of these the most investigated *OCT4* (Matthai et al., 2006; Forte et al., 2009; Bentz et al., 2010), a transcription factor (Loh et al., 2006), is associated with the maintenance of pluripotency (Nichols et al., 1998) and self-renewal in embryonic stem cells and germ cells

(Niwa et al., 2000). As in the mouse, the variable expression of Oct-4 has been reported for immunostaining and RT-PCR analysis in the human endometrium across the menstrual cycle (Matthai et al., 2006). The occasional stromal Oct-4 expressing cell was detected (Matthai et al., 2006) but Oct-4^+ epithelial cells were not detected. Conversely, endometrial epithelial SP cells (reviewed in following paragraph) demonstrated expression of Oct-4 mRNA (Cervello et al., 2010). In adults, the expression of *OCT4* is controversial, with reports of its exclusive expression in stem cells (Takeda et al., 1992; Jiang et al., 2002; Tai et al., 2005) conflicting with its detection in differentiated cells (Zangrossi et al., 2007). It has been suggested that the function of *OCT4* is different in embryonic and adult stem cells (Zangrossi et al., 2007), thus explaining discrepancies in data. Alternatively, the discrepancy of Oct-4 results could be due to the different isoforms examined (Monk et al., 2008; Wang and Dai, 2010). Thus, due to the uncertainty of Oct-4 as a marker of adult stem cells, the *in vivo* transplantation of putative endometrial stem cells becomes imperative.

These data further support the presence of putative stem/progenitor cells in the endometrium. However, without *in vitro* and *in vivo* assessment, it is unknown whether these putative stem/progenitor cells are functional and makes the identification of such stem cell markers meaningless without this confirmation.

1.8.2.4 Side population cells

In the endometrium, epithelial stem cells have been identified through the sorting of SP cells (Kato et al., 2007). These were primarily localised to vascular endothelial cells and epithelial glands in the basalis by expression of Bcrp1 protein (Tsuji et al., 2008; Masuda et al., 2010). SP cells were enriched in the $CD9^-/CD13^-$ (epithelial and stromal markers respectively) fraction, showed long-term (<24 weeks) survival ability *in vitro* (Kato et al., 2007) and had six-fold greater colony-forming efficiency than non-SP cells (Tsuji et al., 2008). In culture, SP cells differentiated to produce gland ($CD9^+$)-like and stroma ($CD13^+$)-like cells in three-dimensional Matrigel cultures (Kato et al., 2007). In contrast, non-SP cells senesced

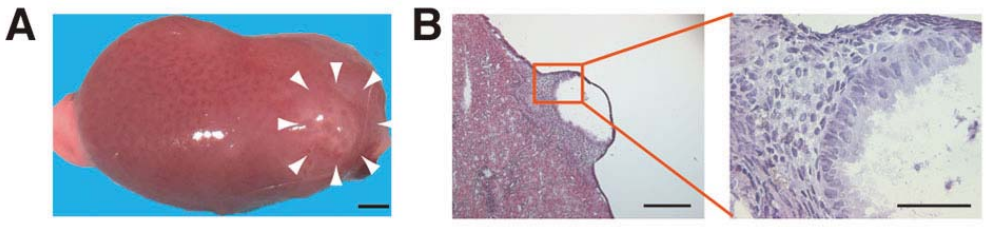


Figure 1.8: Recreation of the human endometrium by SP cells in immunodeficient mice (A) The xenograft located under the mouse kidney capsule (arrow heads). (B) Hematoxylin and eosin staining of the same lesion indicated. Same lesion at higher magnification (outlined in red). Reproduced with permission from Masuda et al. (2010). *PLos ONE* 5: e10387. doi:10.1371/journal.pone.0010387

within 1–3 months. When transplanted under the kidney capsule, SP cells formed epithelial and stromal endometrial-like tissue albeit at a low reconstitution efficiency (Cervello et al., 2010; Masuda et al., 2010).

Although the SP technique enables prospective isolation, the endometrial fraction sorted in most studies has been a heterogeneous one (Tsuji et al., 2008; Masuda et al., 2010) thus a marker/s specific for one stem/progenitor cell type is preferrable. In addition, SP cells differentiated into adipogenic, osteogenic (Cervello et al., 2010) and endothelial lineages indicating that SP cells predominantly consist of mesenchymal and endothelial cells (Cervello et al., 2010; Masuda et al., 2010) and suggests that this technique may not be suitable for isolating a purified endometrial epithelial population.

1.8.2.5 *In vivo* reconstitution of endometrial tissue

An important *in vivo* study demonstrated endometrial reconstruction by transplanting unfractionated human endometrial cells (ie. a mix of epithelial and stromal) under the mouse kidney capsule of NOD/SCID/γ_c^{null} (NOG) immunodeficient mice (Masuda et al., 2007). Reconstructed tissue recapitulated the hierarchical architecture of endometrial and myometrial-like layers. Vessels of the mouse kidney parenchyma were chimeric with both human and mouse cells, suggesting a functional circulatory system had been established to support the endome-

trial construct (Masuda et al., 2007). The reconstructed endometrial tissue was functional as it responded to estrogen and progesterone by undergoing decidualisation. Upon progesterone withdrawal, shedding epithelium suggested that menstruation was taking place (Masuda et al., 2007). The above body of evidence supports the existence of epithelial and mesenchymal stem/progenitor cells in the human endometrium.

1.8.3 Prospective identification of endometrial mesenchymal stem-like cells

Although there is compelling evidence for human endometrial epithelial stem/progenitor cells, their elucidation has been hampered by lack of specific markers for their prospective isolation and characterisation. Unlike the epithelial fraction where no such marker exists, much progress has been made in the prospective identification of endometrial mesenchymal stem cells (eMSC). Screening with a number of potential markers and testing of functional activity was required before two putative markers were identified (Schwab et al., 2008). The co-expression of two perivascular markers CD146 and PDGF-Rβ identified a small population of cells that were clonogenic and underwent self-renewal (Schwab and Gargett, 2007). This was a six-fold enrichment of colony-forming endometrial stromal cells compared to previous reports of unfractionated endometrial stromal cells and a 17-fold over the double negative population (CD146^{-}PDGF-Rβ^{-}) (Chan et al., 2004; Schwab and Gargett, 2007). Clonally derived eMSC exposed to specific induction media underwent multi-lineage differentiation into adipogenic, myogenic, chrondrogenic and osteoblastic cells (Schwab and Gargett, 2007). Although convincing *in vitro* data, transplantation of the CD146^{+}PDGF-Rβ^{+} eMSC into murine models in future will further strengthen the case that these markers prospectively isolate eMSC.

1.8.4 Potential role of epithelial stem/progenitor cells in gynaecological disorders

Common gynaecological disorders associated with abnormal endometrial epithelial proliferation include endometriosis, adenomyosis and endometrial cancer (Gargett and Chan, 2006; Gargett, 2004, 2007). It is hypothesized that stem cells may play an important role in the initiation or pathophysiology of these disorders (Gargett and Chan, 2006; Gargett and Masuda, 2010). Alterations in the number or location of stem cells and changes in uterine regulatory factors may have a role in contributing to endometrial disease.

1.8.4.1 Endometrial cancer

Endometrial cancer (EC) is the most common gynaecological malignancy with 142,000 women worldwide affected per year and an estimated mortality rate of 42,000 annually (Amant et al., 2005). Approximately 10% of patients are considered to have inherited the disease while 90% develop sporadic disease (Ryan et al., 2005; Di Cristofano and Ellenson, 2007). As with other carcinomas, EC is thought to arise from a step-wise accumulation of genetic alterations in cellular regulatory pathways which results in dysfunctional cellular growth (Ryan et al., 2005). Alterations in microsatellite loci or specific mutations in *K-ras*, *HER2* and *PTEN* have been implicated with EC (Ryan et al., 2005). Accumulating evidence suggests that stem cells may be responsible for the initiation and progression of EC and offers an explanation for the functional heterogeneity observed in solid tumours (Reya et al., 2001; Visvader and Lindeman, 2008; Hubbard and Gargett, 2010).

Cancer stem cells (CSC) are defined as a subset of tumour cells with the capacity to self-renew and proliferate extensively to form new tumours (Reya et al., 2001; Visvader and Lindeman, 2008). There have been parallels identified between normal stem cells and malignant CSC (Reya et al., 2001; Pardal et al., 2003). Like normal stem cells, CSC also exhibit the ability to self-renew however their acquired mutations results in the neoplastic

proliferation of primitive progenitors (Reya et al., 2001; Pardal et al., 2003) or enables their escape from the regulatory control of the niche, fueling hyperplasia and tumorigenesis (Li and Xie, 2005). Small populations of SP and clonogenic cells have been identified in EC cell lines and cells from fresh tumour tissue (Friel et al., 2008; Hubbard et al., 2009; Kato et al., 2010). Notably, reduced expression of differentiation markers, long-term proliferation, self-renewal and differentiation into cells of the mesenchymal lineage were observed (Kato et al., 2010). Interestingly, chemotherapeutic drugs had little affect on the viability of CSC SP and may account for why some patients have resistence to chemotherapy (Friel et al., 2008). It can also explain why relapse occurs since not all cancerous cells have been eradicated from the patient and CSC remain (Friel et al., 2008). Tumour-initiating cells were identified in endometrial carcinoma and cell lines following transplantation of isolated single cell suspensions, that produced tumors which retained the original histological phenotype of the primary EC (Hubbard et al., 2009) and also following successive transplants (Friel et al., 2008), demonstrating self-renewal *in vivo*. Lack of PTEN staining in harvested tumours correlated to observations in parent tumours and suggested the clonal origin of tumours (Hubbard et al., 2009). These data strongly suggest a role for CSC in the development and progress of EC.

1.9 Endometriosis

Endometriosis is a gynaecological disorder in which uterine endometrial tissue grows in an ectopic location outside the uterus (Giudice and Kao, 2004). It is an estrogen-dependent chronic disease affecting women of reproductive age that can occasionally be asymptomatic or more likely presents with pain and/or infertility (Rogers et al., 2009). Its estimated prevalence is between 6–10% of the female population (Eskenazi and Warner, 1997) and approximately 50% in the infertile (Meuleman et al., 2009). There is unfortunately no permanent cure for endometriosis and its pathophysiology is not well understood. Following hormonal and

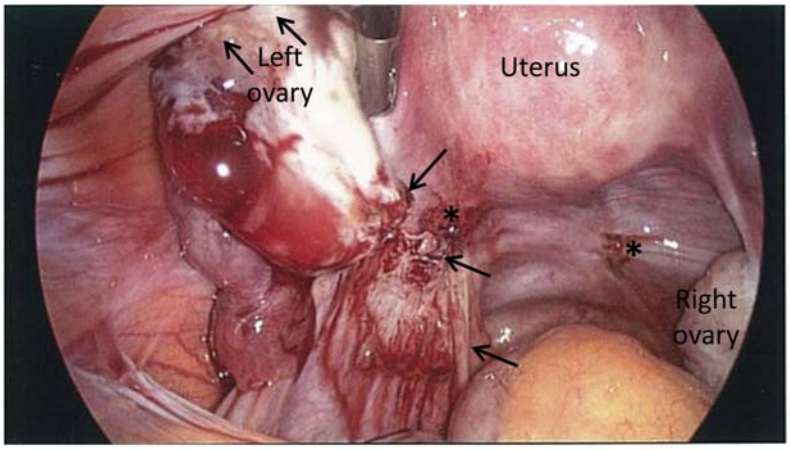


Figure 1.9: Laparoscopy photo of the peritoneal cavity of a patient with severe endometriosis. The left ovary is bound to the peritoneal wall by adhesions. Arrows point to adhesions and *, marks endometriotic lesions. Courteously taken by Dr. Gareth Weston, 2010.

surgical treatment, recurrence rates are high (Meuleman et al., 2009; Berlanda et al., 2010; Vercellini et al., 2010). Endometriosis is a substantial economic burden and healthcare cost to society, costing an estimated US$22 billion in treatment annually in the USA and indirect costs such as loss in work productivity and income (Gao et al., 2006; Meuleman et al., 2009).

1.9.1 Types

Endometriosis is commonly found in the pelvic peritoneum, and to a lesser degree on the ovaries (referred to as endometrioma) and the rectovaginal septum (Bazot et al., 2004; Giudice and Kao, 2004). Endometriotic lesions formed can be invasive and adhesions that develop confine or immobilize the movement of organs in the peritoneal cavity (Giudice and Kao, 2004) (Fig 1.9).

It has been suggested that peritoneal, ovarian and rectovaginal endometriosis should be considered separate entities (Nisolle and Donnez, 1997) with different pathogenesis (Nap et al., 2004). However, their manifestation results in a similar array of symptoms including dyspareunia, dysmenorrhea, dyschezia and chronic pelvic pain (Giudice and Kao, 2004; Mounsey et al., 2006; Attar and Bulun, 2008).

1.9.2 Aetiology, pathogenesis and evidence

Despite being the most common gynaecological disorder, the underlying mechanism of endometriosis is poorly understood. There are many theories regarding its pathogenesis with the most common being the retrograde menstruation and implantation theory. The coelomic metaplasia theory explains some forms of endometriosis while other factors contributing to pathogenesis include altered cellular immunity and genetic alterations.

1.9.3 Retrograde menstruation and implantation theory

Since the 1920s, Sampson's retrograde theory of the pathogenesis of endometriosis has been accepted although no concrete evidence has confirmed it. In its essence, the theory states a small portion of endometrium is shed into the peritoneal cavity during menstruation where it establishes an ectopic implant that responds to hormones and proliferates on subsequent cycles (Sampson, 1927).

1.9.3.1 Evidence from animal models of retrograde menstruation

Animal models have been developed to investigate evidence supporting Sampson's theory. Following transplantations of human immortalised endometriosis cells, endometriotic lesions were found in the peritoneal cavity of nude mice (Banu et al., 2009). The induced lesions consisted of developed or developing glands lined with epithelial cells, surrounded by stromal cells. Numerous blood vessels and capillaries had formed around the endometriotic glands (Banu et al., 2009). Conservation of human and mouse histoarchitecture was identified, demonstrating that human endometriotic cells were responsible for inducing peritoneal endometriosis in the mouse (Banu et al., 2009). Transplantations of human endometrial cells in the kidney capsule of immunocompromised mice resulted in abundant human-derived vessels in endometrial reconstructs that invaded the mouse kidney parenchyma to connect and form a functioning circulation system (Masuda et al., 2007). Mice were treated with estrogen and

progesterone to stimulate menstrual changes in reconstructed endometrium. Macroscopic observations identified large blood-filled cysts on grafts, similar to red endometriotic lesions that consisted of glandular structures on microscopic inspection (Masuda et al., 2007). These data demonstrate ectopic endometrial cells are responsive to hormonal stimulation (Masuda et al., 2007), forming endometriotic lesions (Masuda et al., 2007; Banu et al., 2009) and suggest that endometrial derived endothelial progenitors/cells may have a unique role in the pathogenesis of endometriosis (Masuda et al., 2007; Maruyama et al., 2010; Masuda et al., 2010).

The baboon model of endometriosis has provided insight into retrograde menstruation with prevalence, laparoscopic appearance and histology similar to humans (D'Hooghe et al., 1991). Furthermore, the primate model offers the advantage of longitudinal study that is not ethical with humans (D'Hooghe et al., 1996). Retrograde menstruation was found more frequently in baboons with spontaneous endometriosis (83%) than controls (51%) (D'Hooghe et al., 1996), in agreement with the 76–90% reported in women (Halme et al., 1984; Liu and Hitchcock, 1986). Collection of blood-stained peritoneal fluid was most common (94%) during menstruating phases of the cycle (D'Hooghe et al., 1996), however the number of endometrial cells in peritoneal fluid were not accounted for.

1.9.3.2 Role of peritoneal fluid in the theory of retrograde menstruation

In humans, the peritoneal fluid functions to facilitate mobility of viscera within the peritoneal cavity and is in constant contact with mesothelial cells that line the cavity (Haney et al., 1981). The fluid is thought to be an exudation of the ovary, its volume fluctuating in response to endogenous and exogenous sex hormones (Koninckx et al., 1980), just as endometriotic lesions are stimulated by ovarian hormones (Mounsey et al., 2006). Reports comparing volume of peritoneal fluid have been conflicting with some studies finding significant differences observed between controls and endometriosis women (Haney et al., 1981) and others not (Kruitwagen et al., 1991). A contributor to this discrepancy is the inter-individual variability

in volume which has been reported to be between 5 ml to >200 ml during ovulation (Koninckx et al., 1998). Inspection of peritoneal fluid from women with endometriosis for colour and transparency resulted in reports describing a straw-colour, to those containing red blood cells (Haney et al., 1981; Scheenjes et al., 1990; van der Linden et al., 1995). However, there were no differences in the colour of peritoneal fluid from endometriosis and control women as both commonly contained red blood cells (Scheenjes et al., 1990; van der Linden et al., 1995). These indicate that the collection of hemorrhagic peritoneal fluid is not restricted to women with endometriosis as it is found in most women.

1.9.3.3 Presence of human endometrial cells in the peritoneal cavity

In humans the cellular content of peritoneal fluid has been examined. A higher number of endometrial cells were found in peritoneal fluid of women with endometriosis than those without (Bartosik et al., 1986). Moreover, intact gland structures presumably of endometrial origin were also observed (Bartosik et al., 1986; van der Linden et al., 1995). However, cells could be cultured from peritoneal fluid of women with and without endometriosis (Kruitwagen et al., 1991). Endometrial cell colonies capable of proliferating were identified in both groups, with no differences between the numbers of cell colonies observed (Kruitwagen et al., 1991). Of particular note, another study reported the absence of endometrial epithelial cells from the peritoneal fluid of women without endometriosis (Willemsen et al., 1985). To recreate a retrograde menstrual effect, the investigators performed uterine-tubal flushings and cells from these washings were collected and cultured to observe formation of endometrial epithelial colonies (Willemsen et al., 1985). Similarly, endometrial cells were absent from peritoneal fluid collected from women with endometriosis, however following uterine flushing, endometrial cells were obtained (Bartosik et al., 1986). Therefore, the procedure of uterine-tubal irrigation may flush endometrial cells into the peritoneal cavity, but is not necessarily representative of peritoneal fluid. Although these findings demonstrate endometrial fragments with proliferative capacity can be carried into the peritoneal cavity via retrograde reflux (Willemsen

et al., 1985) these studies leave some questions unanswered. Why were endometrial cells not always found in the peritoneal fluid of women with endometriosis? (Bartosik et al., 1986).

The presence of endometrial cells in peritoneal fluid could also be cycle-dependent. Peritoneal fluid/flushings were collected during the early proliferative (Willemsen et al., 1985; Bartosik et al., 1986; Kruitwagen et al., 1991; van der Linden et al., 1995), or secretory phase (Bartosik et al., 1986) and suggests that rapid clearance was mediated by immune cells present in peritoneal fluid, or the endometrial fragments/cells had already attached to the peritoneal wall. It is surprising that no sampling was undertaken during menstruation when efflux occurs.

1.9.3.4 Role of macrophages and cytokines in the theory of retrograde menstruation

Peritoneal macrophages are cells involved in the immune surveillance of the peritoneal cavity. Macrophages secrete growth factors and cytokines which play a major role in the initiation, propagation and regulation of immune and inflammatory responses (Siristatidis et al., 2006). Given their predominance in peritoneal fluid, macrophages and their factors have been investigated as having mitogenic effects on the growth of endometrial cells (Haney et al., 1981; Loh et al., 1999). These factors could potentially explain the growth of endometrial cells in the peritoneum and subsequent development of endometriotic lesions. The co-culture of macrophages isolated from the peritoneal fluid of women with endometriosis and endometrial epithelial or stromal cells revealed a significant increase and enhancement in proliferation respectively, in comparison to controls (Loh et al., 1999). It was also reported that peritoneal fluid enhanced the autologous growth of eutopic and ectopic endometrial cells from women with endometriosis (Braun et al., 2002). Notably, peritoneal fluid from controls significantly suppressed the growth of autologous eutopic endometrial cells (Braun et al., 2002) indicating differences between peritoneal fluid composition in control and endometriosis women. Immunostaining of endometriotic lesions revealed macrophages were responsible for producing BLyS cytokine (B lymphocyte stimulator (Hever et al.,

2007)) which is involved in B cell development (Schiemann et al., 2001; Darce et al., 2007). Higher levels of CD68 (macrophage marker), NRL-MACRO and HAM56 (markers of later macrophage differentiation) were detected in peritoneal fluid of women with endometriosis compared to controls (Montagna et al., 2008). Tumor necrosis factor-α (TNF-α), interleukin-6, and interleukin-β were also highly expressed in macrophages obtained from women with endometriosis (Montagna et al., 2008). These data further support the important role macrophages play in endometriosis.

Although the majority of women experience retrograde menstruation (Halme et al., 1984; Liu and Hitchcock, 1986) and the presence of endometrial cells in peritoneal fluid (Bartosik et al., 1986; Kruitwagen et al., 1991), only 6-10% develop endometriosis (Eskenazi and Warner, 1997), suggesting that there are other predisposing factors responsible for the development of endometriosis.

1.9.4 Coelomic metaplasia theory

The coelomic metaplasia theory suggests that peritoneal endometriosis results from metaplastic changes in the peritoneal mesothelium. This metaplasia is induced by retrograde reflux of endometrial stroma rich in growth factors and cytokines (Matsuura et al., 1999). Published reports have demonstrated normal mesothelium changes into endometrial glandular cells by immunohistochemistry studies (Nakamura et al., 1993; Mai et al., 1997), however more definitive data are required. The presence of endometriotic lesions in peritoneal cavities of premenarcheal girls indicates that retrograde reflux alone does not explain aetiology (Marsh and Laufer, 2005). If endometriosis resulted from mesothelial metaplasia, much higher rates of pleural endometriosis should be observed as the lining of the pelvic and abdominal regions of the peritoneal cavity are the same (Taylor and Lebovic, 2009), however this is not the case.

1.9.5 Immune and inflammatory system

Others have suggested that endometriosis results from an underlying pathology of the immune system (Hever et al., 2007), particularly a lack in adequate surveillance mechanisms (Lebovic et al., 2001). Microarray analysis of endometriotic lesions revealed 53 genes with altered expression associated with the immune response, inflammatory diseases and cell to cell signaling (Hever et al., 2007). Upon establishment of endometriotic lesions, secretion of proinflammatory mediators begins (Giudice and Kao, 2004). Cytokines (interleukins-1 and -8, TNF-α, and interferon-γ) induce chemotactic factors (including RANTES, regulated on activation, normal T expressed and secreted) which recruit macrophages and T cells into the peritoneal cavity resulting in a cascade of inflammatory reactions associated with endometriosis (Giudice and Kao, 2004). Abundant numbers of plasma cells and macrophages have also been detected in endometriotic lesions (Hever et al., 2007), further supporting the case for aberrant immune and inflammatory systems in endometriosis patients.

Women with endometriosis have also been reported to have higher rates of autoimmune diseases (multiple sclerosis, rheumatoid arthritis, Sjgren's syndrome, systemic lupus erthematosus) (Sinaii et al., 2002). Investigation into the potential shared molecular and cellular pathways of endometriosis and rheumatoid arthritis revealed rheumatoid arthritis genes *CCL21* and *HLA-DRB1* were associated with both conditions (Sundqvist et al., 2010). To demonstrate the effect of cytokines, human endometrial cells from eutopic and ectopic endometrium were cultured with autologous peritoneal fluid (Braun et al., 2002). Addition of a soluble TNF-α-receptor inhibitor reduced or eliminated the proliferative effect of peritoneal fluid on cultured endometrial cells from women with endometriosis (Braun et al., 2002). However these findings did not clarify whether the effects of peritoneal fluid on endometrial cell proliferation were exclusively due to the actions of TNF-a or reflect a more complex interaction between multiple stimulatory factors (Braun et al., 2002).

1.9.6 Genetics

Endometriosis is regarded as a complex disorder with environmental and genetic components contributing to the development and progression of disease. Genetic contribution has been investigated in twins as well as incidences in first-, second-, and third- degree relatives (Treloar et al., 1999; Nouri et al., 2010). Genome-wide linkage analysis of Australian twins revealed monozygotic twins had a higher risk ratio (2:1) of developing endometriosis than dizygotic twins (Treloar et al., 1999). Researchers also reported a higher incidence of endometriosis in first-degree relatives of women with endometriosis (Coxhead and Thomas, 1993; Moen and Magnus, 1993). However, this is in disagreement with Nouri et al. (2010), who reported a tendency towards an increased risk but no statistical significance suggesting that genetic predisposition is a contributing factor but not cause for the development of endometriosis.

Many candidate gene polymorphisms have been evaluated in women with endometriosis (Montgomery et al., 2008; Tempfer et al., 2009). These studies have been focused on genes involved in inflammatory mediators (cytokines, nitric oxide, adhesion molecules, human leukocyte mediators, RANTES), sex hormones and hormone regulators (estrogen, progesterone and androgen receptor), metabolic enzymes, vascular function and tissue remodeling regulators (vascular endothelial growth factor, epidermal growth factor receptor and endostatin, angiotension-I-converting enzyme, matrix metalloproteinases, a2-HS glycoprotein, plasminogen activator inhibitor-1) (Montgomery et al., 2008; Tempfer et al., 2009). Results however are conflicting which could be attributed to the small sample size studied (Treloar et al., 1999; Montgomery et al., 2008). Therefore studies with greater power are required (Montgomery et al., 2008).

To investigate the mechanisms responsible for pathogenesis, an immunocompromised mouse model of endometriosis has enabled investigation of gene pathways involved in endometriotic lesion formation (Hull et al., 2008). Microarray analysis of endometriotic lesions revealed four key pathways (cell injury and necrosis, inflammation, tissue remodeling/repair,

and ras-mediated oncogenesis), indicating the communication between endometrial and mesothelial cells and highlighting the role of the immune and inflammatory systems in development of endometriosis (Hull et al., 2008). In addition, the expression profile of microRNAs (miRNAs) was detected by microarray in paired ectopic and eutopic endometrial tissues (Ohlson Teague et al., 2009). miRNAs are naturally occurring posttranscriptional regulatory molecules regulating gene expression and may play a role in endometriotic lesion development (Ohlson Teague et al., 2009). Twenty-two miRNAs were identified by their differential expression and putatively regulate the expression of 2340 genes (Ohlson Teague et al., 2009), a subset of which were previously reported (Hever et al., 2007; Hull et al., 2008; Ohlson Teague et al., 2009). These studies indicate the genetic complexity of endometriosis and future investigations into the specific role of miRNAs as well as immune and inflammatory pathways are required.

1.9.7 Endometrial-peritoneal cell interactions

Mesothelial cells line the peritoneal cavity and their interactions with endometrial cells have been investigated using tissue/cells to remodel endometriotic lesion formation *in vitro* (Witz et al., 1999; Nair et al., 2008). It was suggested, that an intact peritoneal lining prevents the adherence of endometrial fragments (Groothuis et al., 1999). However, others have contradicted this finding and reported the adhesion of endometrial cells to intact mesothelium (Witz et al., 1999). These differences could be accounted by the power in sample size (n=3 and n=15, respectively). Importantly, all cases of adhesion were dependent upon endometrial stromal cells, suggesting that stromal cells were required for the initial step of invasion into the mesothelium (Witz et al., 1999). Single cell suspensions of endometrial cells (epithelial and stromal) were reported to have invaded through modeled peritoneum within 6–12 hours (Nair et al., 2008). By 24 hours, endometrial cells had invaded and spread on the underside of the modeled peritoneum, suggesting that the transition from attachment to invasion is likely to

be too rapid to allow observation *in vivo* (Nair et al., 2008). The increased expression of gene products implicated in invasion and metastasis (CD44, extracellular signal-related kinase, colony stimulating factor-1, c-fms and c-Met) were detected when endometrial cells were co-cultured with peritoneal mesothelial cells (PMC), but not conditioned medium from PMC (Nair et al., 2008).This suggests the importance of direct cell to cell contact, as occurs between endometrial and mesothelial cells in the development of peritoneal endometriotic lesions. It was also reported that endometrial stromal cells isolated from different women varied significantly in adherence to PMC, suggesting that the altered binding rates might explain the difference between lesion establishment in women with and without endometriosis (Lucidi et al., 2005). However, these studies need to be repeated using endometrial and mesothelial cells collected from women with and without endometriosis to form a true comparison.

1.9.8 Diagnosis and Treatment

Laparoscopy and laparotomy remain the gold standards in the surgical assessment and diagnosis of endometriosis (Giudice and Kao, 2004), since diagnosis relies on visual inspection of the peritoneal cavity. Classification of disease is visually determined based upon the 1985 revised American Fertility Society Classification of Endometriosis Staging System (ASRM, 1997). However some patients are asymptomatic (Mihalyi et al., 2010) or premenarcheal (aged 8.5–13 years) (Marsh and Laufer, 2005) in whom surgery is avoided and can result in a diagnostic delay of 8–11 years (Husby et al., 2003; Ballard et al., 2006). Plasma biomarkers have been investigated as a non-invasive diagnostic (Mihalyi et al., 2010), with an added economical benefit in comparison to surgery (Rogers et al., 2009). The combined use of biomarkers and advanced statistical analysis has enabled a high sensitivity and high specificity detection of endometriosis (Mihalyi et al., 2010). Furthermore, investigators were able to distinguish between control and minimal-mild endometriosis and validation studies are currently underway (Mihalyi et al., 2010).

Current treatment of endometriosis relies on a two pronged approach of medical and surgical therapy. Medical strategies are usually initiated to relieve pain and limit menstruation. These come in the form of gonadotropin-releasing hormone agonists, oral contraception, progestins, androgens and non-steroidal anti-inflammatory agents (Ozawa et al., 2006; Giudice, 2010). These aim to lower estrogen levels interfering with the hypothalamic pituitary gonadal axis inducing a menopausal state, however long-term side effects (eg. osteoporosis) limit this approach. More importantly, the hypoestrogenic state results in endometrial atrophy (Giudice, 2010) affecting the fertility of patients who are usually of child bearing age. Often, medicinal therapy provides little benefit because of tissue distortion and invasion of lesions into the bowel and other organs. Surgery currently remains the best option (Szendei et al., 2005). Surgical treatment involves the excision and ablation of endometriotic peritoneal lesions, excision or drainage of endometriomas, resection of rectovaginal nodules, lysis of adhesions and interruption of nerve pathways (Giudice, 2010). It is the preferred first-line approach for women who wish to become pregnant or for those who do not respond to medicinal therapies (Jackson and Telner, 2006). However, surgery only provides temporary relief and reoccurrence of endometriosis and symptoms occurs with a reported 44.7% of patients continuing to experience pain post-operatively (Gao et al., 2006).

1.9.9 Endometrial stem/progenitor cells in endometriosis

It has been proposed that endometrial tissue fragments shed during menstruation contain stem/progenitor cells that are capable of initiating ectopic growth and development of endometriosis (Leyendecker et al., 2002; Gargett, 2004; Gargett and Chan, 2006; Gargett, 2007; Sasson and Taylor, 2008; Gargett and Masuda, 2010; Maruyama et al., 2010) (Fig 1.10).

Studies have examined the use of menstrual blood as a source for mesenchymal stem cells for regenerative medicine applications (Cui et al., 2007; Meng et al., 2007; Hida et al., 2008; Patel et al., 2008). Rapidly growing cells from menstrual blood were cultured with a

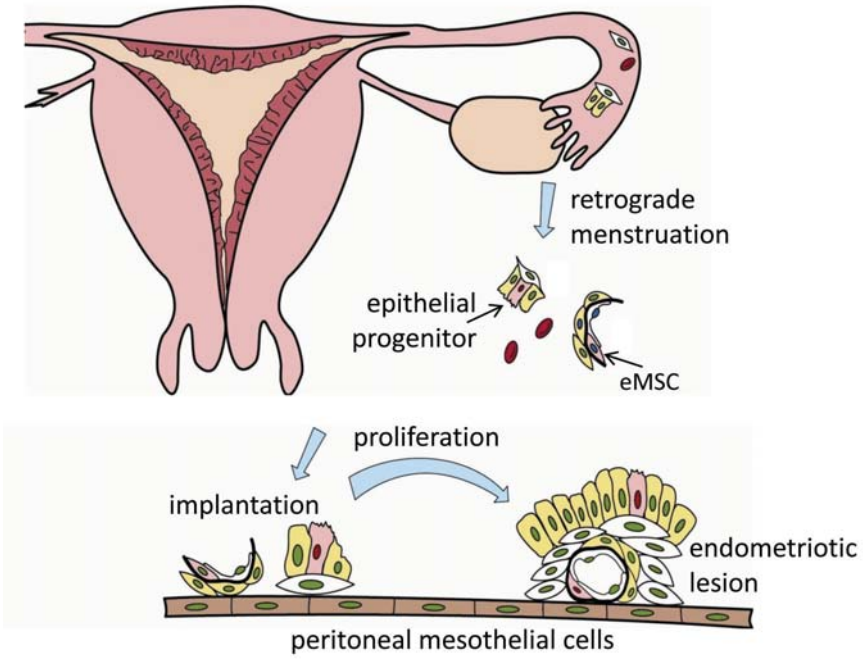


Figure 1.10: Schematic diagram of the possible role endometrial epithelial progenitor cells may play in endometriosis. It is hypothesized that retrograde menstruation effluxes endometrial stem/progenitor cells together with their niche cells into the peritoneal cavity where they establish endometriotic lesions in women who develop endometriosis. eMSC, endometrial mesenchymal stem cell. Adapted with permission from Gargett and Guo (2010) published in Endometriosis Current Management and Future Trends by Jaypee Brothers and Gargett and Masuda (2010) *Mol Hum Reprod* 16(11): 818-34.

doubling (PD) every 19.4 hours (Meng et al., 2007). Adherent cells expressed mesenchymal stem cell markers including CD29, CD59, CD73, CD41a, CD44, CD90 and CD105 (Meng et al., 2007). Of particular interest was the detection of hTERT and Oct-4 expression (Meng et al., 2007), although data was not shown. Several endometrial stromal cell lines were established by single cell plating and in one case these maintained a stable karyotype for up to 68 PD (Meng et al., 2007), higher than that reported for bone marrow HSC (Gronthos et al., 2003; Kern et al., 2006). Cultured menstrual blood MSC-like cells (mbMSC; also termed endometrial regenerative cells, menstrual blood stromal stem cells or menstrual blood-derived mesenchymal cells) had a fibroblastic appearance and expressed similar markers (CD29, CD44, CD73, CD90, CD105) to clonogenic and $CD140b^{+}CD146^{+}$ endometrial

MSC-like cells, and similarly lacked hemopoietic (CD14, CD34, CD45), endothelial (CD31) and other stem cell (CD133, Stro-1) markers (Cui et al., 2007; Meng et al., 2007; Hida et al., 2008). There were several notable differences in cell surface phenotype on mbMSC observed in different laboratories for c-kit, CD13 and CD9 markers (Cui et al., 2007; Patel et al., 2008). This disparity is likely due to the heterogeneity of the menstrual blood cell population, differences in cell selection processes and culture media used (Gargett and Masuda, 2010).

mbMSC were capable of differentiating into all three germ layer lineages; mesodermal (myocyte, osteocyte, endothelium, adipocyte, cardiomyocyte), ectodermal (neuronal) and endodermal (hepatic, pancreatic, respiratory epithelium) after culturing with commercially available induction media *in vitro* (Meng et al., 2007; Patel et al., 2008). Following five days of co-culture with murine fetal cardiomyocytes, approximately half of mbMSC were synchronously beating (Hida et al., 2008) demonstrating differentiative capacity. Action potentials obtained to determine functionality demonstrated 'pacemaker potentials'. mbMSC injected into the right thigh of NOG mice were detected between myocytes in the muscle bundles and in interstitial tissue 1–3 weeks later implying extensive migration had occurred (Cui et al., 2007). When transplanted into a mouse model of musclar dystrophy, expression of dystrophin (skeletal muscle protein) was detected in mbMSC without any treatment or induction (Cui et al., 2007). These data suggest that shedding endometrium is capable of myogenic transdifferentiation and further work is needed to develop this potential cell-based therapy for patients with Duchenne muscular dystrophy. These studies support the presence of clonogenic cells in menstrual blood, however these studies have focused upon stromal cells, with data for epithelial cells surprisingly absent. Possible reasons for this could be that mesenchymal cells have overgrown the epithelial cells (Musina et al., 2008) or that they were not retrieved, suggesting that epithelial progenitors remain within the basalis that is not shed during menstruation (Gargett and Masuda, 2010). However further investigations are required to determine whether epithelial progenitors are shed during menstruation.

Gene profiling of endometriotic tissues indicated differential expression of genes (*SALL4*,

UTF1, *TCL1*) associated with early development and oncogenesis when compared to normal endometrium (Forte et al., 2009). Notably, *ZFP42*, a gene expressed during human testes development was expressed exclusively in 25% of endometriotic tissues examined (Forte et al., 2009). The expression of self-renewal and pluripotency genes *OCT4*, *KFL4*, *BMI1* were detected however the same was reported for normal endometrium (Forte et al., 2009). Flow cytometric analysis of eutopic and ectopic endometrial cell lines established from patient samples, detected similar expression of mesenchymal markers CD9, CD29, CD44, CD90 and CD105 (Kao et al., 2010). Although PD and colony-forming efficiency were similar between groups, ectopic endometrial cells showed greater migration and invasion abilities compared to eutopic eMSC (Kao et al., 2010). Endometriotic stromal cells were seeded in scaffolds and transplanted into the SCID mouse and assessed for invasion ability *in vivo*. After harvesting, inspection of the scaffolds revealed formation of irregular shaped spheres that contained stromal tissues supported by blood vessels, indicating that ectopic eMSC induced angiogenesis (Kao et al., 2010), similar to endometriotic lesions (Laschke and Menger, 2007). Expression of CD49f was greater in ectopic than eutopic endometrial cells (90.5% and 73.7% respectively; Kao 2010).

Interestingly, CD49f, an integrin (α-6) transmembrane protein, has been used as a marker of mammary gland (Stingl et al., 2006; Lim et al., 2009) and prostate epithelial stem cells (Litvinov et al., 2006). It has been suggested that CD49f contributes to the invasive capacity of oncogenic cells (Kim et al., 2009; Ohara et al., 2009) which might explain the invasive properties of effluxed endometrial stem/progenitor cells in the peritoneal cavity, although investigations are needed to confirm this. Previous studies have examined the invasiveness of ovarian endometriotic lesions *in vitro* (Forte et al., 2009) however this may not be representative of peritoneal endometriosis, as these should be considered separate entities with differing pathogenesis (Nisolle and Donnez, 1997; Nap et al., 2004). Nonetheless, these ovarian endometriotic mesenchymal cells demonstrated clonogenic and proliferative behavior similar to previous reports in eMSC and mbMSC (Cui et al., 2007; Meng et al., 2007; Hida

et al., 2008; Gargett et al., 2009) and should not be disregarded.

Currently, human endometrial epithelial progenitor cells have only been identified retrospectively using stem cell function assays (Chan et al., 2004; Schwab et al., 2005; Gargett et al., 2009). To further characterise these cells, the discovery of a specific markers or a defined set of markers for their prospective isolation is essential. Using such a marker, the location of the endometrial stem cell niche would be uncovered and the role of epithelial progenitors in the development of disorders such as endometriosis could be elucidated. To date, the presence of clonogenic cells in shedding endometrium and peritoneal fluid from women with and without endometriosis undergoing menstruation has not been investigated. There is a lack of data comparing normal and hyperproliferative endometria. Functional assays and molecular analysis should be completed to further understand the role endometrial stem/progenitor cells may play and the pathways involved, which would lead to the substantial improvement of clinical treatments. For instance, medicinal therapies could target ectopic endometrial stem/progenitor cells and specifically remove them from the peritoneal cavity without the need for invasive and repeated surgery, thus offering patients greater relief and recovery from a painful and recurrent disorder such as endometriosis.

1.10 Hypotheses

1.10.1 Overall hypothesis

Human endometrium contains a small population of epithelial progenitor cells which can be identified by surface markers and are present in shedding endometrium and peritoneal fluid of women with endometriosis.

1.10.2 Specific hypotheses

Chapter 2 A unique pattern of surface markers on endometrial epithelial progenitor cells distinguishes them from their progeny, the mature epithelial cells.

Chapter 3 HER3 is a marker that identifies epithelial progenitor cells in the human endometrium.

Chapter 4 Shedding endometrium contains viable endometrial stem/progenitor cells that are effluxed into the peritoneal cavity during menstruation in women with endometriosis.

1.11 Aims

1.11.1 Overall aim

To identify markers for the prospective isolation of human endometrial epithelial progenitor cells.

1.11.2 Specific aims

Chapter 2 To screen the endometrium for reactivity to a panel of novel and known antibodies and generate a priority list for subsequent assessment of their ability to identify epithelial progenitor cells.

Chapter 3 To assess HER3 as a marker for the prospective isolation of human endometrial epithelial progenitor cells.

Chapter 4 To determine if endometrial stem/progenitor cells are present in the peritoneal fluid of menstruating women with and without endometriosis and determine if they express putative and known endometrial stem/progenitor cell markers.

CHAPTER 2

Identifying candidate markers of human endometrial epithelial progenitor cells

2.1 Introduction

Epithelial stem/progenitor cells have been shown to exist in the human endometrium (Chan et al., 2004). It is suggested that these stem/progenitor cells are responsible for the maintenance of the highly regenerative endometrial tissue (Prianishnikov, 1978; Padykula, 1991; Gargett, 2007). The endometrium is shed and rapidly renewed (Novak and Te Linde, 1924; Ludwig and Spornitz, 1991) over 400 times during a women's reproductive age (Eaton et al., 1994; Jabbour et al., 2006), following mensturation, parturition and in post-menopausal women taking estrogen-only hormone replacement therapy (Feeley and Wells, 2001; Gargett, 2007).

The endometrium forms the mucousal lining of the uterus and is located adjacent to the myometrial muscle layer. Columnar epithelial cells form the luminal surface and glandular epithelium and these extend from the surface down to the endometrial-myometrial interface. The endometrium is functionally divided into two regions, the upper functionalis and lower basalis. The upper functionalis consists of epithelial glands which are loosely supported by

stroma. The basalis consists of branching glands that have penetrated the functionalis and are supported by dense stroma and vessels. During menstruation, the functionalis is shed and regenerates in the following cycle from the remaining basalis (Ludwig and Spornitz, 1991). It is hypothesised that stem/progenitor cells which reside in an endometrial niche located in the basalis region are responsible for this regeneration (Prianishnikov, 1978; Padykula, 1991; Gargett, 2007).

Despite the evidence for epithelial progenitor cell activity in the human endometrium, there are no means of prospectively isolating this rare population for further characterisation. The identification of specific markers would allow epithelial progenitor cells to be distinguished from their mature differentiated progeny. Subsequently, the role of epithelial progenitor cells could be examined in proliferative disorders such as endometriosis and endometrial cancer. In organs such as the prostate and breast, surface markers have been identified that enable the prospective isolation of epithelial stem cells (Stingl et al., 2001; Welm et al., 2002; Clarke et al., 2003; Richardson et al., 2004; Stingl et al., 2006; Lawson et al., 2007; Miki et al., 2007; Tang et al., 2007). Whilst there is no single universal marker of stem cells, the use of multiple markers has been used to purify other epithelial stem cells capable of differentiating into their tissue of origin (Stingl et al., 2006; Lawson et al., 2007; Miki et al., 2007).

Flow cytometry and immunohistochemistry are the most common techniques used for phenotypic cell analysis. Both are complementary. Flow cytometry allows for multiple markers to be identified at a single cell level, as well as quantifies the expression of each individual marker within a cell population. Immunohistochemistry allows for morphologic visualisation and localisation of labelled cells in reference tissue. In an effort to identify markers for the prospective isolation of endometrial epithelial progenitor cells a panel of novel and known antibodies to cell surface markers were screened using these two techniques, a strategy developed to produce a priority list of candidate progenitor cell markers.

2.2 Materials & Methods

2.2.1 Human Tissues

Endometrial tissues were collected from ovulating women (n=33, aged 42.1±1.2 years, range 28–52, Table 2.1) and non-ovulating women (n=2, aged 38.5) undergoing hysterectomy (n=30) or curettage (n=5) who had not received hormonal treatment three months prior to surgery. Twelve patient samples were obtained during the proliferative phase, 18 from the secretory phase of the menstrual cycle, three were poorly differentiated and two were inactive. Full thickness endometria with 5 mm attached myometrium or curettes were collected in Collection Medium (DMEM/F12 with HEPES; 1% Antibiotics, both Invitrogen, Carlsbaad, CA, USA; 2% fetal calf serum, Gibco/Invitrogen) and processed within 2–24 hr, or frozen in OCT Tissue Tek (Sakura Finetek Co., Tokyo, Japan) on dry ice and stored at -80 °C until required. The stage of the menstrual cycle of the samples was assessed by histological examination according to experienced pathologists according to well established criteria for the normal menstrual cycle (Noyes et al., 1975) and was obtained from pathology reports. Post-menopause (inactive endometrium) was defined by ≥12 consecutive months of no menstruation (amenorrhea). Ethics approval was obtained from the Monash Medical Centre Human Research and Ethics Committee B. Informed written consent was obtained from each patient.

2.2.2 Preparation of single cell suspensions of human endometrial cells

Human endometrium was scraped off and/or mechanically dissociated using scalpel blades into Bench Medium (DMEM/F12 with HEPES, 10% Newborn Calf Serum, 1% antibiotics; Invitrogen) and underwent first digestion in Collagenase type 3 (2.5mg/ml; Worthington Biochemical Corporation, New Jersey, USA)/PBS, DNaseI (1mg/ml; Worthington Biochemical Corporation)/PBS at 37 °C. The suspension was mechanically dissociated for 10–15 min

Age (yrs)	Stage	HYST/CUR	Indication for surgery	Expt
28	ES	CUR	Chronic pelvic pain	FC
30	S	CUR	Infertility	FC
31	ES	CUR	Infertility	FC
31	IN	HYST	Leiomyoma	IHC
32	P	HYST	Menorrhagia	FC
34	P	HYST	Chronic endometritis	IHC
36	MS	HYST	Benign endometrial polyp	FC
36	P	HYST	Menorrhagia	IHC
37	P	HYST	Bulky uterus	FC
38	MP	HYST	Menorrhagia	IHC
39	MS	CUR	Infertility	FC
39	S	HYST	Menorrhagia	FC
40	P	CUR	Infertility	FC
41	S	HYST	Prolapse	FC
42	S	HYST	Menorrhagia, leiomyoma	IHC
43	ES	HYST	Menorrhagia, leiomyomata	FC
43	S	HYST	Endometritis	IHC
44	PD	HYST	Leiomyomas	FC
44	LS	HYST	Leiomyoma	IHC
45	PD	HYST	Leiomyomas	FC
45	S	HYST	Menorrhagia, adenomyosis	FC
45	P	HYST	Leiomyomas	IHC
45	P	HYST	Leiomyomata	IHC
46	LS	HYST	Adenomyosis	FC
46	IN	HYST	Leiomyoma	IHC
47	ES	HYST	Leiomyomas	FC
47	P	HYST	Leiomyomata	FC
48	S	HYST	Leiomyomata	IHC
49	LS	HYST	Menorrhagia	FC
49	S	HYST	Leiomyoma	FC
51	P	HYST	Menorrhagia	FC
51	PD	HYST	Myometrial leiomyoma	FC
51	P	HYST	Prolapse	IHC
52	P	HYST	Endometriosis	FC
52	S	HYST	Menorrhagia, leiomyoma	IHC

Table 2.1: Sample characteristics. Menstrual stages: proliferative (P), early proliferative (EP), secretory (S), mid-secretory (MS), late secretory (LS), poorly differentiated (PD), inactive (IN). Endometrial tissue from a hysterectomy (HYST) or curettage (CUR). Tissue sample was used for flow cytometry analysis (FC) or immunohistochemistry (IHC).

intervals and progress was monitored using a light microscope. After 60 min, enzyme activity was stopped by dilution (1:3) with Bench Medium. The cell suspension was filtered through 40 and 35μm strainers (BD Falcon, New Jersey, USA). The first digestion and filtration allowed for a crude seperation between single stromal cells and epithelial glandular fragments. Glandular fragements collected by the strainer were further digested in Collagenase type 2 (4mg/ml; Worthington Biochemical Corp)/PBS and DNaseI/PBS at 37 °C. After 20–30 min, enzyme activity was stopped by dilution (1:3) with Bench Medium. Digested cells were filtered through 40 and 35μm strainers to remove any remaining cell aggregates. Erythrocytes and dead cells were removed from the suspension using Ficoll-Paque density gradient centrifugation medium (GE Healthcare, Uppsala, Sweden) centrifuged at 1500 rpm for 15 min (Schwab et al., 2008). The cells at the Ficoll-Paque interface were collected and washed twice in Bench Medium. Leukocytes were removed from the cell suspension using CD45 magnetic bead separation (Dynabeads, Invitrogen, Carlsbad, USA) according to the manufacturer's instructions (Chan et al., 2004; Schwab and Gargett, 2007).

2.2.3 Immunolabelling endometrial cells for flow analysis

Single colour flow cytometry was used to screen freshly isolated endometrial cells (n=21) for reactivity to a panel of supernatant antibodies (n=24, Table 2.2) generously donated by Dr Hans-Jörg Bühring (University Clinic of Tübingen, Tübingen, Germany). These antibodies had reactivity to stem cells or cancer cells in other tissues and for this reason they were selected for the panel. This is the first study to examine these antibodies in endometrium.

Antibody clone	CD	Other Name	Isotype	Reactivity	Reference
1/4 C4		Frizzled-10 receptor (Fz10)	IgG2a	Syncytiotrophoblasts of placental villi	
1B4C3		HER3/erbB3	IgG2a	Tumor cells	
24D2	CD340	HER2/erbB2	IgG1	Tumor cells, BM-MSC	Blood 1995; 86:1916-23.
2D1D12		HER3/erbB3	IgG1	Tumor cells	
4FR6D3	CD344	Frizzled- 4 receptor (Fz4)	IgG1	Neuronal progenitor cells	
51D6		DDR1	IgM	Epithelial cells	J Immunol 2005; 174:6490-8
57D2			IgG1	Neural progenitor cells	Haematologica 2003; 88:126-33
67A4	CD324	Ecadherin	IgG1	Erythroid, epithelial	J Cell Biol. 1995; 131:243-9.
67D2	CD164		IgG1	SC, leukemic, basophil activator marker	Blood 2000; 95:3113-24. J Immunol 2000; 165:840-51.
97A6	CD203c		IgG1	Basophil, mast, secretory glands in endometrium	Blood 2001; 97:3303-5.
9A3G9		Breast carcinoma protein (Br Ca)	IgG1	BM-MSC	Ann N Y Acad Sci. 2009; 1176:124-34.
BV10	CD135	Flt3/Flk2	IgG1	SC, leukemic cells	Blood 1997; 90:111-25
CUB1	CD318		IgM	Tumor cells, SC, CDCP1+ cells	Stem Cell 2004; 22:334-43.
F93C2C1		Embryonic kidney	IgG1	BM-MSC	Ann N Y Acad Sci. 2007; 1106:262-71
P3C4	CD172a		IgG2a	SC, myeloid cells, MSC	Haematologica. 2003; 88(2):126-33.
W3C4E11	CD349	Frizzled-9 receptor (Fz9)	IgM	Placenta/BM-MSC, parenchymal cells	J Cell Physiol. 2010; 225(1):123-31.
W3C5 pRb		Retinoblastoma protein	IgG2b	BM-MSC	
W3D5 pRb		Retinoblastoma protein	IgG2a	BM-MSC	Blood 1999; 94:3633-43.
W5C4 pRb		Retinoblastoma protein	IgG2b	BM-MSC	Ann N Y Acad Sci. 2007; 1106:262-71
W6B3C1	CD133		IgG1	HSC, NSC, endothelial SC	
W7C5 pRb	CD109		IgG1	MSC	Ann New York Acad Sci. 2003; 996:227-30.
W7C6 pRb		Retinoblastoma protein	IgG2a	BM-MSC	Blood 1999; 94:3633-43.
W8B2B10		Mesenchymal stem cell antigen-1 (MSCA-1)	IgG1	BM-MSC	Ann N Y Acad Sci. 2007; 1106:262-71 Stem Cells Dev. 2010; 19: 669-77
DOR5A2B4		δ-Opioid receptor	IgG2a	Tumor cells, SC	

Table 2.2: The panel of supernatant antibodies used for screening of endometrial cell reactivity. CD, cluster differentiation

To develop flow cytometry protocols for the primary supernatant antibodies, three dilutions (100μl/ml, 250μl/ml and 500μl/ml) were dispensed into tubes containing endometrial cells ($5x10^4$–$1x10^5$). Isotype controls consisted of isotype control antibody (10μl/ml, 25μl/ml and 50μl/ml) and Bench Medium to recreate a supernatant to parallel the primary antibodies. EpCAM, an epithelial cell marker (5.4μg/ml; mouse anti-human IgG_1; clone Ber-EP4; Dako Cytomation, Glostrup, Denmark) was used as a positive control. Endometrial cells were washed in Flow Buffer (2% FCS/PBS), blocked with goat serum (5%, Sigma, St. Louis, MO USA) and incubated in a total volume of 100μl with each of the 2–24 antibody supernatants or controls (IgG_1, IgG_{2A}, IgG_{2B}, IgG_M) in separate tubes (BD Falcon) for 45 min at 4 °C. Tubes were subsequently incubated with sheep anti-mouse IgG PE conjugated secondary antibody (Dako Cytomation) for 30 min at 4 °C. Samples were either immediately analysed or fixed with 2% paraformaldehyde for analysis within 24 hours. Similar analysis results were obtained if cells were fixed or unfixed.

Data was acquired using a MoFlo flow cytometer (DakoCytomation, Fort Collins, CO, USA) and Summit version 5.0.1.3804 (Dako Colorado, Inc.; USA). Cells were selected for analysis by electronically gating the forward and side scatterplot thus removing dead cells and debris (Fig 2.3).

Following this a minimum of 5,000 events were collected for each sample and the percentage of cells reacting with the antibody, above background levels of isotype control, was determined (Fig 2.4). Results are shown as mean $\pm$ SEM, unless otherwise stated.

2.2.4 Immunohistochemistry

Uterine tissues were cryosectioned (Leica, Wetzlar, Germany) at 5μm onto silane (Sigma Aldrich, St. Louis, U.S.A) coated glass slides (Menzel GmbH + Ko Kg, Braunschweig, Germany) and stored at -80 °C until required. Frozen uterine sections were thawed to room temperature (RT) then fixed in acetone for 2 min at 4 °C. Sections were subsequently incubated

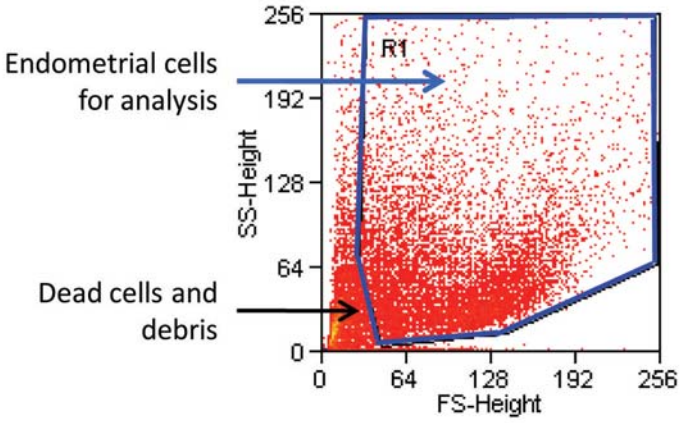


Figure 2.3: Scatterplot of human endometrial epithelial cells. Forward (FS) and side scatter (SS) are used to determine cell size and complexity respectively. Dead cells, debris and erythrocytes can be excluded using electronic gating. Only cells within the gated region R1 (outlined blue) were analysed.

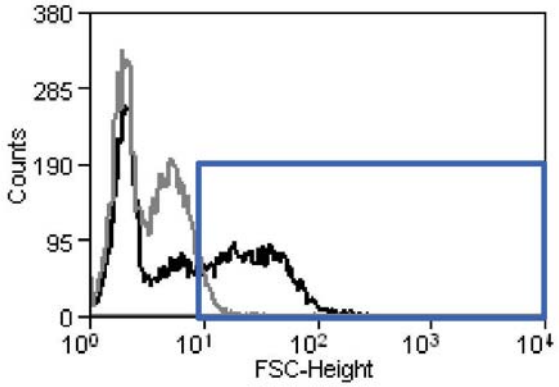


Figure 2.4: Overlay flow cytometry histogram of endometrial cells reacting with supernatant antibody (black) and isotype control (grey). Positive cells are within the blue box, which is adjusted until there are <2% of isotype control events. To calculate percentage of positive cells, background level is subtracted from marker antibody.

with 0.3% hydrogen peroxide (Orion Laboratories, Welshpool, Australia) to block endogenous peroxidase and protein blocking agent (Thermo Electron Corp, Pittsburgh, U.S.A) to minimise non-specific antibody binding for 10 min each at RT. Primary and isotype control antibodies were diluted in 0.1%BSA/PBS, and incubated for 1 hr at 37 °C or left overnight at 4 °C. Antibodies used were mouse anti-human monoclonal antibody supernatants (H-J.Bühring; Table 2.1). Initial experiments were undertaken to optimise the antibody concentrations required for each supernatant antibody by using 100μl/ml, 250μl/ml and 500μl/ml with matching isotype controls. Mouse isotype IgG_1 (Dako Cytomation), IgG_{2A} (Chemicon, Massachusetts, U.S.A) and IgG_{2B} (Caltag/Invitrogen, Carlsbad, U.S.A) controls were used. Isotype controls consisted of isotype control antibody (10μl/ml, 25μl/ml and 50μl/ml) and bench medium to recreate a supernatant to parallel the primary antibody. EpCAM, an epithelial marker (2.4μg/ml; clone Ber-EP4; Dako Cytomation) was used as a positive control. Sections were subsequently incubated in Biotinylated Streptavidin LSAB +System -HRP Kit (Dako Cytomation) for 30 min at RT. Antibodies were visualised with DAB chromogen (3,3'-diaminobenzidine tablets and urea peroxidase; both from Sigma, St. Louis, U.S.A) for 5 min at RT and counterstained with Harris Hematoxylin Solution (Amber Scientific, Midvale, Australia). Sections were prepared for permanent mounting by dehydration and clearing solvents i.e. three washes in 100% ethanol followed by three washes in Xylene all 2 min each. Slides were mounted using DPX mountant for microscopy (BDH, VWR international Ltd., Poole, U.K) covered by a glass cover-slip (Menzel GmbH + Ko Kg) and examined under a Zeiss Axioskop microscope (Carl Zeiss, Jena, Germany). Images were captured using a Zeiss AxioCam ICc3 camera (Carl Zeiss) and analysed using Axiovision software (Rel 4.6, Carl Zeiss). Stained slides were examined to identify the cellular localisation of antibody marker immunoreactivity and scored according to intensity of epithelial staining - negative (–), weak (+), medium (++), and strong (+++). Heterogeneity of epithelial glandular staining was also noted.

2.2.5 Determining optimal antibody dilution

The supernatant antibodies received from our collaborator Dr Hans-Jörg Bühring, have never been used by our laboratory. Thus, to utilise the antibodies for the prospective isolation of endometrial epithelial progenitor cells using flow cytometry, optimisation of these antibodies had to intially be carried out.

To determine optimal antibody dilution for flow cytometry, single colour analysis was done on proliferative and secretory stage endometrial samples to minimise any variability between different stages of the menstrual cycle. An example is anti-CD203c (Fig 2.5A). Overlay histograms show that during proliferative stage a 100μl/ml dilution identifies more CD203c $^+$ cells compared to 250μl/ml, although a similar precent was observed for 500μl/ml. Similarily during the secretory stage, a 100μl/ml dilution identified more CD203c $^+$ cells compared to 250μl/ml. Therefore, 100μl/ml dilution of the CD203c antibody supernatant was considered optimal for use in flow cytometry irrespective of the menstrual cycle stage.

To determine the optimal antibody dilution for immunohistochemistry, staining was done on full thickness endometrial sections to examine epithelial immunoreactvity in the functionalis and basalis regions. Using anti-CD203c as an example, little staining was observed for the 100μl/ml dilution (Fig 2.5B). Little difference was observed between 250μl/ml and 500μl/ml dilutions and the lower 250μl/ml was selected as the optimal dilution for immunohistochemistry.

Optimisation of the flow cytometry protocol was performed for all supernatant antibodies. Only antibodies that were immunoreactive with endometrial cells by flow cytometry were then optimised and localised in the endometrium by immunohistochemistry to form a short list of candidate endometrial epithelial progenitor markers.

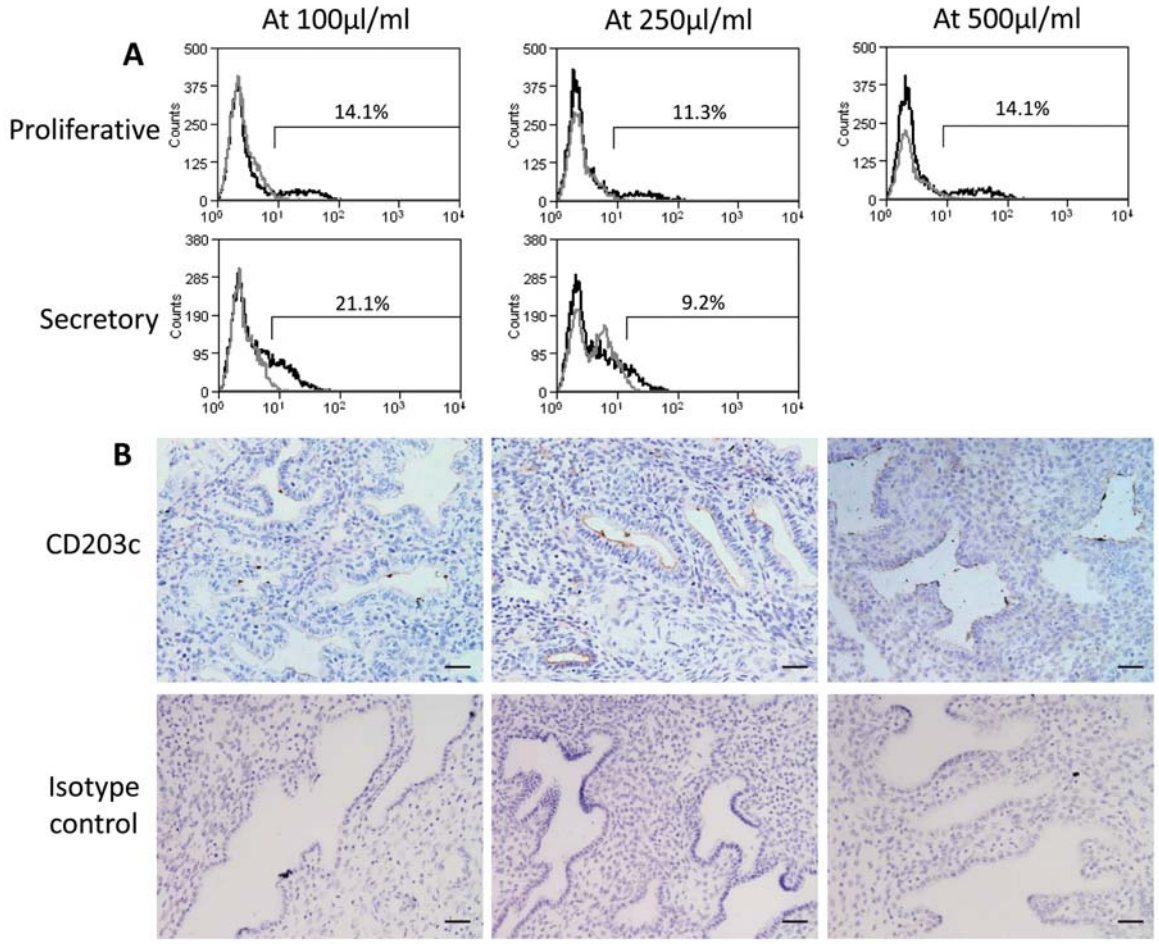


Figure 2.5: Optimisation of antibodies in the panel, CD203c as an example. (A) To determine endometrial reactivity, endometrial single cell suspensions were screened by flow cytometry with a supernatant antibody and corresponding isotype control at three dilutions (100μl/ml, 250μl/ml and 500μl/ml) during proliferative and secretory stage of the menstrual cycle. (B) To reveal location of reactivity, full thickness uterine sections were immunostained with supernatant antibody at three dilutions (as above; top row) and corresponding isotype control (bottom row). Scale bar: 100μm.

2.3 Results

Twenty-four known and novel markers were assessed for expression in human endometrial epithelial cells to generate a priority list of potential epithelial progenitor cell markers. Screening for reactivity was firstly examined by flow cytometry and immunhohistochemistry to determine location of expression in the uterus and identify specificity for epithelial cells.

2.3.1 Screening for endometrial reactivity to the antibody panel using flow cytometry

Initial screening on endometrial single cell suspensions with the 24 supernatant antibodies by single-colour flow cytometry was done to determine expression in the endometrium. Twenty of the 24 novel and known antibodies reacted with endometrial cells. At different stages of the menstrual cycle, a large variation was observed between patients (Fig 2.6). Endometrial single cell suspensions were unfractionated and included epithelial, stromal, endothelial and vascular smooth muscle cells. Thus, localisation of antibody had to be determined in endometrial tissue using immunohistochemistry.

2.3.2 Immunohistochemistry of novel and known antibodies in the endometrium

Immunohistochemistry was undertaken on uterine sections for the cellular localisation of antibodies that reacted with endometrial cells by flow cytometry (20 out of 24; Table 2.7). Full thickness uterine sections were examined to compare for any staining differences between functionalis and basalis. Three dilutions of supernatant antibody were compared and one was selected as optimal as described in 2.2.5. Five out of six antibodies (CD133, CD203c, E-cadherin, HER2, and HER3) that were specific for glandular epithelium, interestingly had stronger staining in the basalis region compared to functionalis. E-cadherin was the only

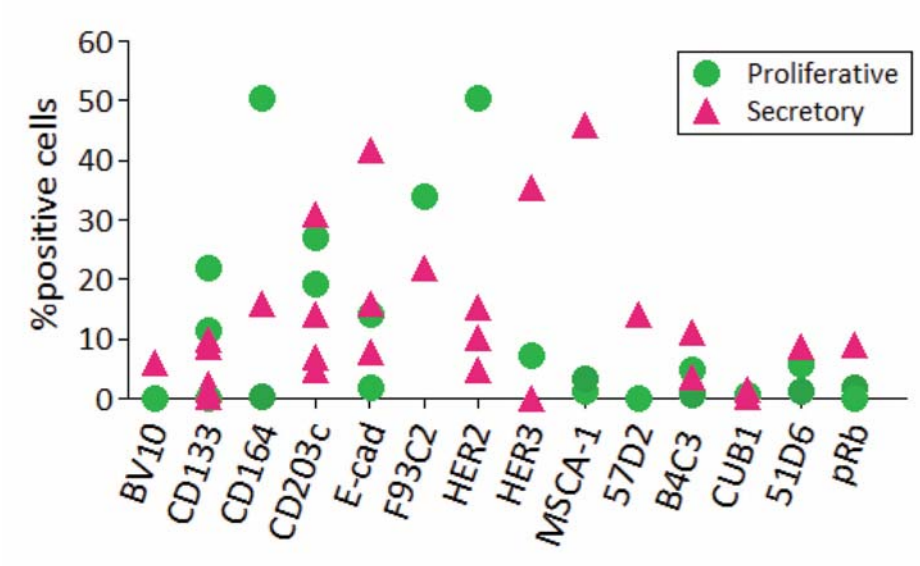


Figure 2.6: Screening of unfractionated human endometrial cells for immunoreactivity to a panel of antibody markers. Results of single colour flow cytometry analysis. Data is shown as a scatterplot, with each dot representing an individual sample. 2–7 samples were examined for each antibody and were at the proliferative or secretory stage of the menstrual cycle.

marker constitutively expressed by all luminal and glandular epithelial cells regardless of the stage of menstrual cycle and in the inactive endometrium. Differences in staining between two clones of HER3, D1D12 and B4C3, were observed. However, these differences did not affect flow cytometry analysis as similar percentages of cells was obtained (Fig 2.6). Nevertheless, immunostaining observations indicated that B4C3 was not an appropriate clone for this study and was not further pursued. HER3 D1D12 (herein referred to as HER3) exclusively stained the luminal and glandular epithelium irrespective of the stage of the menstrual cycle however intensity was stronger and staining was heterogenous during the secretory stage. Mesenchymal stem cell antigen-1 (MSCA-1) and CD203c had similar levels of expression in luminal and glandular epithelium and were both strongest during secretory stage of the menstrual cycle. CD203c was expressed by glandular epithelium in the inactive endometrium and heterogeneously stained glandular epithelium during menstruation.

Antibody	Dilution (µl/ml)	F vs B	Proliferative		Secretory		Menstrual	Inactive	Cellular reactivity	No. samples
			Luminal	Glandular	Luminal	Glandular	Glandular	Glandular		
CD133	250	b>f	hetero ++	++	-	hetero +	hetero +	hetero +/++	Epith	8
CD203c	250	b>f	++	++	++	+++	hetero +/++	+/++	Epith	9
E-cadherin	250	b>f	++	+/++	++	+/++	++	++	Epith	7
HER2	500	b>f	++	hetero +	++	+/++	-/+	++	Epith	9
HER3 B4C3	500		-	-/+		-/+	ND	ND	Epith	4
HER3 D1D12	500	b>f	+	++	++	hetero +/++	ND	++	Epith	6
MSCA-1	500	f=b	++	++	++	+++	ND	hetero ++	Epith, Peri	7
δ-opioid R	250		-	-	-	-	ND	ND	Stro, Peri	6
57D2	250		-	-	-	-/+	-/+	-	Stro, Myo	9
Br Ca	500		+	-	+	-	ND	ND	Myo, Peri	2
CD109	500		-	-	-	-	-	-	Stro, Peri	4
CD164	500		-	-	-	-	-	-	Stro	8
F93C2	500		-	-	-	-/+	ND	ND	Stro, Myo, Peri	6
pRb W5C4	250		-	-	-/+	hetero -/+	ND	ND	Peri	4
pRb W7C6	500		-	-	-	-	-	-	Myo, Peri	2
BV10	500		-	-/+		+	ND	ND	Neg	6
CUB1	500		-	-	-	-	ND	ND	Neg	2
CD172a	-		-	-	-	-	ND	ND	Neg	4
Frizzled 4-R	-		-	-	-	-	-	-	Neg	4
Frizzled 10-R	-		-	-	-	-	ND	ND	Neg	2

Table 2.7: Immunostaining optimisation and localisation of novel and known antibodies in human endometrium. Dilution, optimal supernatant antibody dilution. Comparisons of staining intensity in basalis (b) to functionalis (f), F vs B. Luminal, luminal epithelium. Glandular, glandular epithelium. Intensity of staining, negative (-), weak (+), medium (++), strong (+++), heterogeneous staining of epithelium (hetero), not done (ND). Cellular reactivity, epithelial (epith), myometrial (myo), negative (neg), perivascular (peri), stroma (stro). Number of human samples at different stages of the menstrual cycle used (No. samples).

MSCA-1 and CD133 both hetrogeneously stained glandular epithelium in the inactive endometrium. Furthermore, CD133 heterogeneously stained the luminal epithelium during proliferative phase and menstruation. MSCA-1 stained epithelial cells as well as perivascular cells, however the heterogeneous staining of glandular epithelium in the inactive endometrium was of interest and it was thus prioritised. Other antibodies (57D2, BrCa, CD109, CD164, σ-opioid R, F93C2, W7C6 pRb, W5C4) stained endometrial stroma, perivascular cells, myometrium or a combination (Fig 2.8). Intensity of staining was classified as outlined in 2.2.4. Each antibody was optimised and tested on different patient samples at different stages of the menstrual cycle and in post-menopausal endometrium in duplicates.

2.3.3 Formation of an antibody priority list

In generating a short list of potential epithelial progenitor markers, data from flow cytometry and immunohistochemistry were taken into account. The process is outlined in a flow chart (Fig 2.9). Twenty-four supernatant antibodies had been selected based upon previous reactivity to stem or cancer cells, however their expression by endometrial cells had to be determined. Initial screening by flow cytometry revealed four antibodies (Fz4, Fz10, CD172a and W5C4) that did not react with the unfractionated endometrial cells and were excluded from further analysis. Twenty endometrial reactive antibodies were immunolocalised on full thickness endometrial tissue. Thirteen antibodies (σ-opioid R, 57D2, 9A3G9, BrCa, BV10, CD109, CD164, CUB1, DDR1, F93C2, Fz9, W3D5, W7C6) reacted to stroma, perivascular cells and/or myometrium were not further investigated. HER3 clone B4C3, did not immunostain endometrial sections as well as clone D1D12 and was excluded from the short list.

Six out of 24 antibodies (CD133, CD203c, E-cad, HER2, HER3, MSCA-1) were specific for glandular epithelia with exception of MSCA-1 which stained epithelial and perivascular cells (section 2.3.8 for justification). Antibodies were ranked according to five points. The most important being whether the antibody had been reported on stem/progenitor cells in

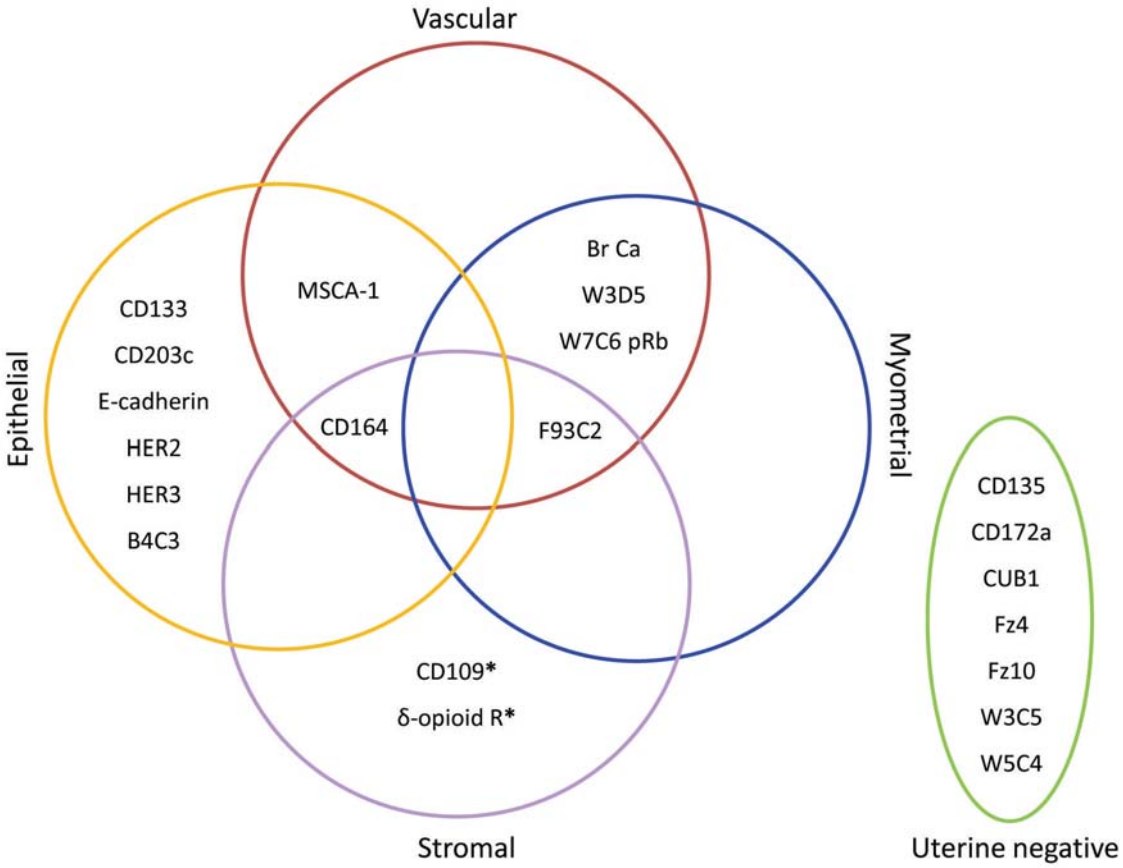


Figure 2.8: Venn diagram showing immunoreactivity of antibody panel with human uterus. *, antibodies immunostained stromal and vascular cells.

other tissues, or been studied in cancer/cancer stem cells. CD133, has been previously used to isolate epithelial stem and cancer stem cells (Richardson et al., 2004; O'Brien et al., 2007; Haraguchi et al., 2008; Vander Griend et al., 2008) and fit this criteria. Similarily, HER2 and HER3 were also highly ranked because of their role in breast and endometrial cancer (Kraus et al., 1989; Rasty et al., 1998; Srinivasan et al., 1999; Ejskjaer et al., 2007; Koutras et al., 2010). Based upon the hypothesis that cancer stem cells exist, these markers associated with tumourgenesis could potentially be expressed on cancer stem cells. Secondly, antibodies that are expressed strongly in the basalis compared to functionalis region, were highly prioritised as the basalis is where the endometrial stem cell niche is thought to be located. Heterogeneous

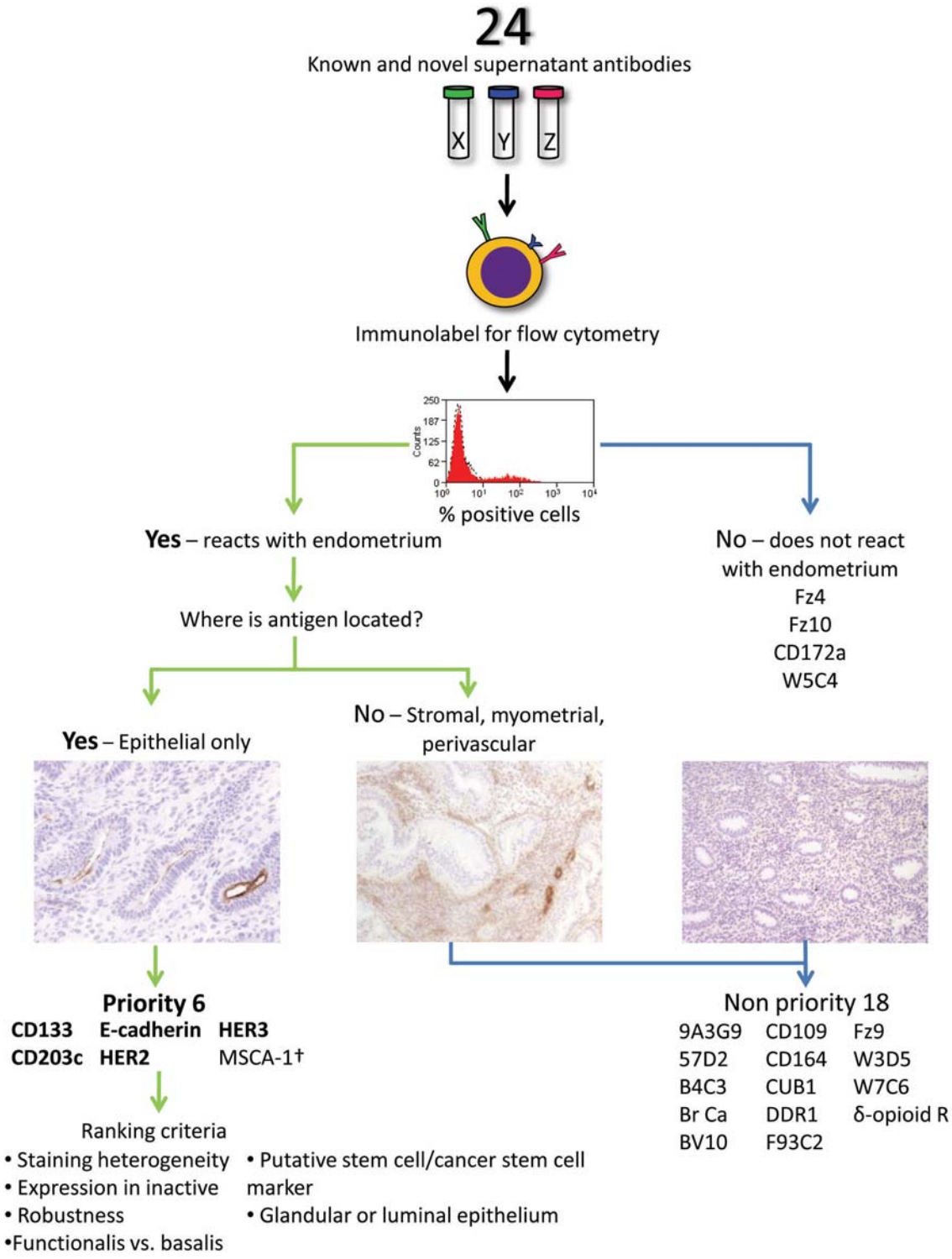


Figure 2.9: Flow chart of generating a short list of candidate endometrial epithelial progenitor markers. †, MSCA-1 immunostained epithelial and perivascular cells.

marker staining of the glandular epithelium was another important consideration. Being a rare population, progenitor cells would not be expected to populate entire epithelial glands thus homogeneous immunostaining is not expected. Heterogeneous staining of epithelium was made more interesting if it was in the inactive endometrium suggesting that these antibody positive cells persist in tissue for a lifetime, a property of adult stem/progenitor cells. Finally, an antibody had to be robust and not luminally secreted (Table 2.10).

Rank	Antibody	Clone	Reasons for rank
1	HER3	D1D12	b>f, hetero, inactive, robust
2	CD133	W6B3	b>f, hetero, inactive, known stem cell marker
3	CD203c	97A6	b>f, hetero, inactive
4	MSCA-1	W8B2B10	b=f, hetero, inactive, known stem cell marker
5	HER2	24D2	b>f, hetero, inactive, association with cancer
6	E-cadherin	67A4	b>f, inactive, enzymatically sensitive

Table 2.10: The short list of six potential endometrial epithelial progenitor markers. Comparisons of staining intensity in basalis (b) to functionalis (f). Hetero, heterogeneous staining.

2.3.4 Antibodies that failed to stain the epithelium

Four out of 24 antibodies (CD172a, Fz4, Fz10 and W5C4) were not immunoreactive with the endometrium by flow cytometry (Fig 2.11A, Suppl Fig 2.18) and thus were not further pursued by immunohistochemistry. The expression of F93C2 was detected by flow cytometry (Fig 2.6) however this novel antibody of unknown specificity was localised in stroma and perivascular cells (Fig 2.11B). Expression of 57D2 was detected on unfractionated endometrial cells by flow ctyometry (Fig 2.6) but immunostaining revealed specificity for myometrial cells (Fig 2.11C). High expression of CD164 was detected by flow analysis (Fig 2.6) however this marker stained epithelial, stromal and perivascular cells in full thickness endometrial sections (Fig 2.11D). CD109 immunoreacted with perivascular and myometrial cells (Fig 2.11E), W3D5 pRb strongly immunoreacted with perivascular cells (Fig 2.11F) and W7C6

was detected by flow analysis (Suppl Fig 2.18) but immunostained stroma and myometrium (Fig 2.11G). δ-Opioid receptor (Fig 2.11H, Suppl Fig 2.18), novel CUB1 (Fig 2.11I, Suppl Fig 2.18) and novel BV10 (Fig 2.11J, Suppl Fig 2.18) were negative with human endometrium.

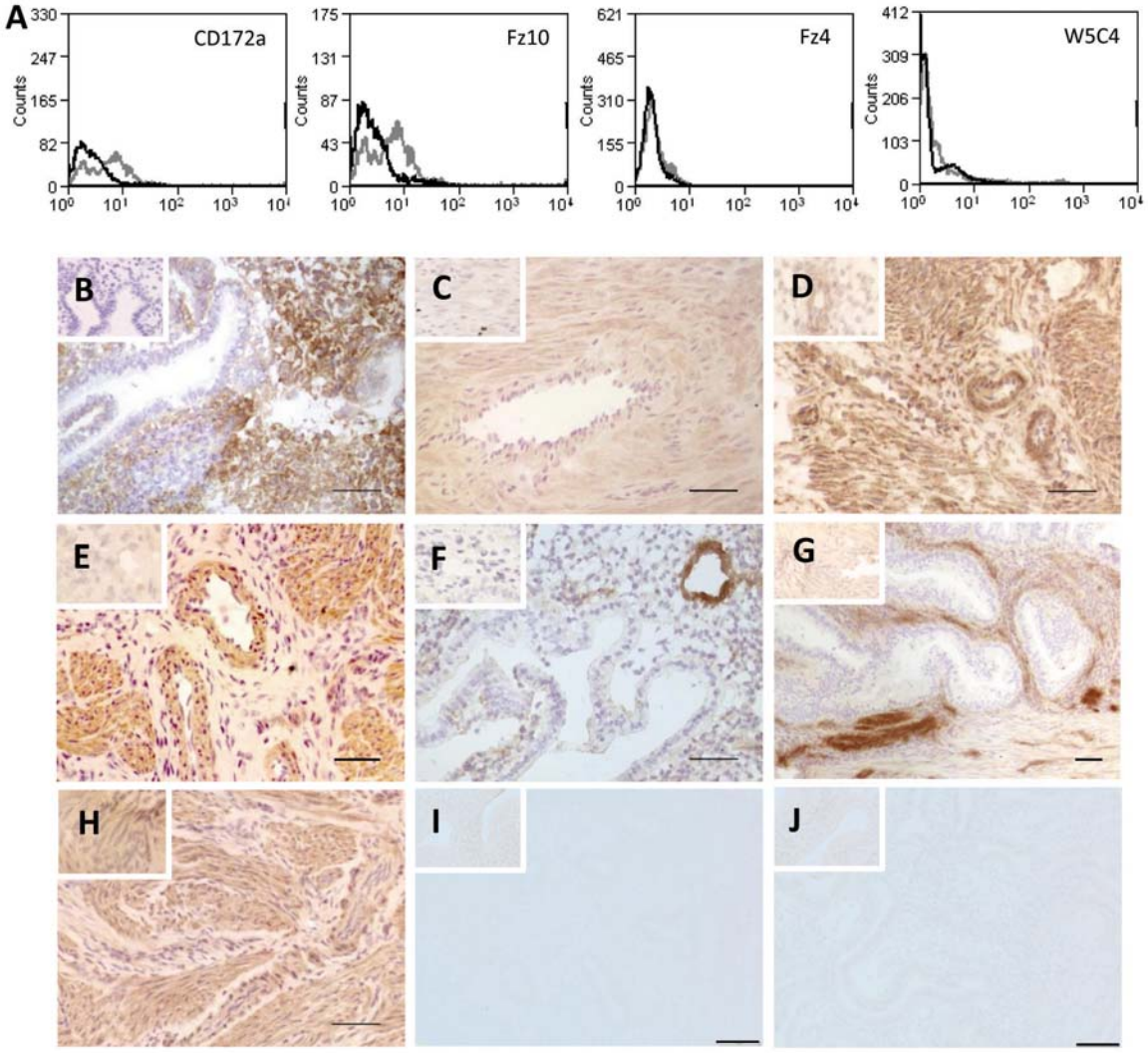


Figure 2.11: Flow cytometry and immunohistochemistry profiles of antibodies not prioritised. (A) Examples of single parameter histograms for CD172a, Fz4, Fz10 and W5C4. Black line: supernatant antibody. Grey line: isotype control. Representative staining of uterine sections (B) F93C2, (C) 57D2, (D) CD164, (E) CD109, (F) W3D5 pRb, (G) W7C6 pRb, (H) δ-Opioid receptor, (I) CUB1 and (J) BV10. (I, J) were not counterstained. Insert: representative section stained with isotype matched control. Scale bar: 100μm.

2.3.5 Antibodies specific for human endometrial epithelium

2.3.5.1 CD133

CD133 (AC133), is a known marker present on epithelial, neural and cancer stem cells in various tissues (Schwartz et al., 2003; Richardson et al., 2004; O'Brien et al., 2007; Haraguchi et al., 2008; Vander Griend et al., 2008; Zhang et al., 2008; Elsaba et al., 2010). CD133 was expressed on $6.7 \pm 4.4\%$ cells during proliferative (n=5) and $4.6 \pm 2.0\%$ during secretory stage (n=5, Fig 2.12A,B). Interestingly, the lower basalis region had stronger staining compared to the upper functionalis (Fig 2.12C,D). Closer inspection revealed heterogeneous staining of luminal and glandular (Fig 2.12E) epithelium during proliferative stage. Expression of CD133 was less during secretory stage, a finding reflected by flow cytometry data. It was interesting to observe the odd singular $CD133^+$ cell in glandular epithelium during secretory stage (Fig 2.12F). Staining in the inactive endometrium was heterogeneous with some areas of the glandular epithelium stronger than others, however the antigen appeared to be secreted into the lumen or was down regulated (Fig 2.12G). The ability of CD133 to prospectively enrich for stem/progenitor cells in colon, prostate and brain (Uchida et al., 2000; Richardson et al., 2004; O'Brien et al., 2007) made it a highly prioritised antibody for the endometrial epithelium.

2.3.5.2 CD203c

CD203c, is a known marker of basophils, mast cells and secretory endometrial glands (Bühring et al., 2001, 2004). The expression of CD203c was 23.2% (mean) during proliferative (n=2) and $14.3 \pm 5.9\%$ during secretory stage (n=4, Fig 2.13A,B). Staining was heterogeneous in the apical membrane of the glandular epithelia in the functionalis (Fig 2.13C) but homogeneous in the basalis (Fig 2.13E) during proliferative stage. Staining of luminal epithelium was a consistent medium intensity during the menstrual cycle (Fig 2.7). In contrast, glandular epithelium showed weaker staining during secretory stage (Fig 2.13D,F) which correlated

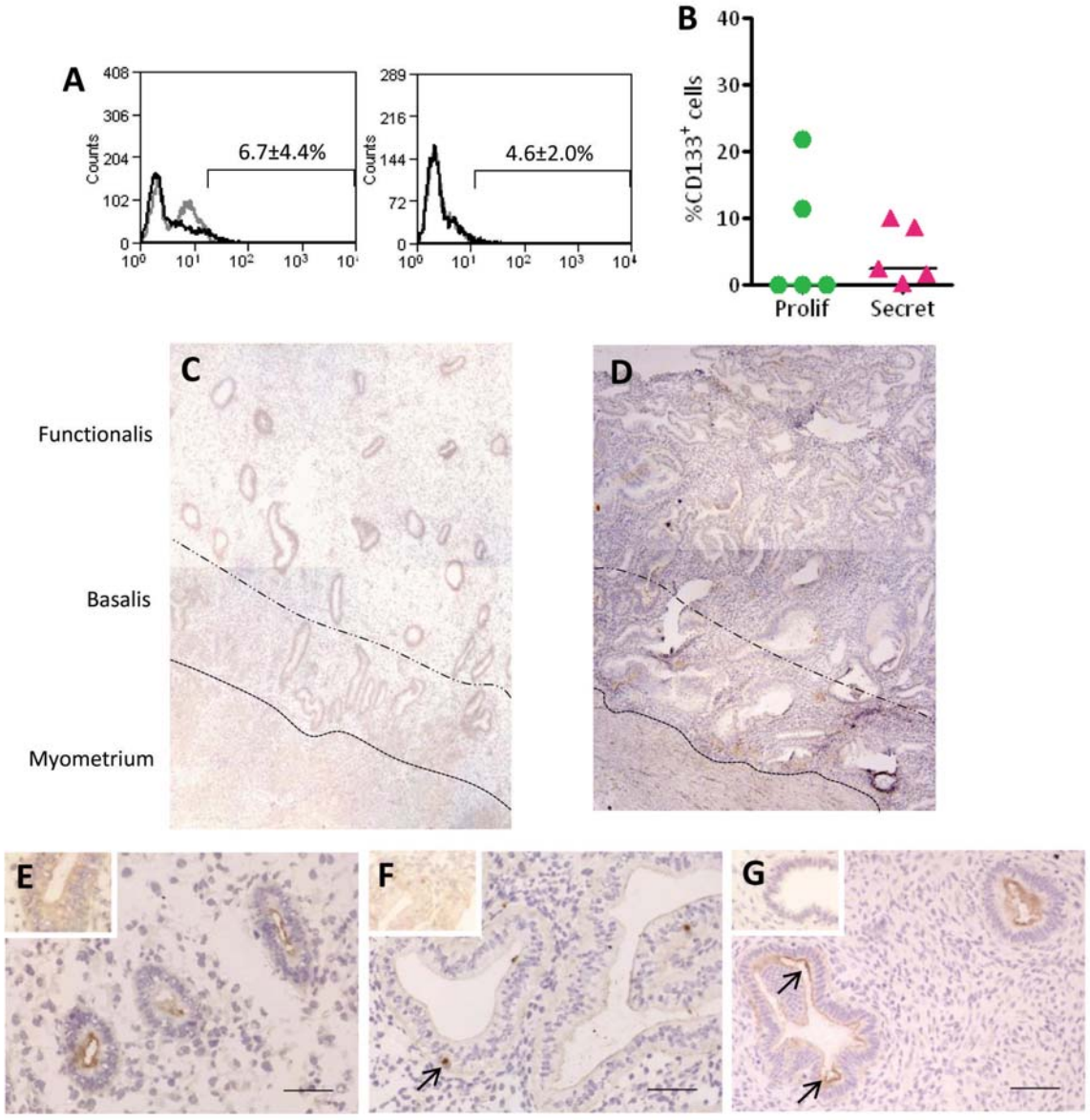


Figure 2.12: CD133 expression in human endometrium. (A) Representative single parameter flow cytometry histogram for CD133, showing percentage of positive cells (mean ± SEM, n=5) during proliferative (left, n=5) and secretory (right, n=5) stages. Black line: supernatant antibody. Grey line: isotype control. (B) Percentage of CD133 $^+$ epithelial cells in proliferative and secretory phase endometrial cell suspensions. Data is shown as a scatterplot, with each dot representing an individual sample. Solid bar represents median. CD133 staining of full thickness endometrial section at (C) proliferative and (D) secretory stages. 2.5X magnification. Representative staining of glandular epithelium during (E) proliferative, (F) secretory and (G) post menopausal endometrium. Inset: representative section stained with isotype matched control. Heterogeneous staining (arrow). Scale bar: 100μm.

with the flow cytometry results (Fig 2.13B). In the post-menopausal endometrium, strong homogeneous staining was observed (Fig 2.13G). Interestingly, stronger expression in glandular epithelial cells in the basalis was observed compared to the functionalis and for this reason CD203c was given priority.

2.3.5.3 E-cadherin

E-cadherin, is expressed by erythropoietic-lineage and epithelial cells (Armeanu et al., 1995; Beliard et al., 1997; Tsuchiya et al., 2006). E-cadherin was expressed on 7.9% cells during proliferative (n=2) and 11.9% cells during secretory stage (n=2, Fig 2.14A,B). Luminal and glandular epithelium were strongly homogeneous immunostained during proliferative stage (Fig 2.14E) and in post-menopausal glandular epithelium (Fig 2.14G) but reduced during secretory stage (Fig 2.14F). The strong E-cadherin immunostaining observed did not correlate with the flow cytometric data, suggesting that the epitope is sensitive to the enzymatic digestion used to prepare single endometrial cell suspensions. Nonetheless, immunostaining of epithelial glands was greater in the basalis compared to functionalis for secretory stage (Fig 2.14C,D) and for this reason E-cadherin was short listed. However, due to its enzyme sensitivity, it was of lower priority.

2.3.5.4 HER2

HER2 (ErbB2/neu), a marker of epithelial cells (Ejskjaer et al., 2005) and bone marrow mesenchymal stem cells (Bühring et al., 2007) also plays a role in epithelial cancers (Gusterson et al., 1988; Borg et al., 1989; Brys et al., 2007; Konecny et al., 2008; te Velde et al., 2009). It was expressed on 7.8% cells during proliferative (n=1) and 13.3 $\pm$ 6.0% cells during secretory stage (n=3, Fig 2.15A,B). Endometrial epithelial cells were weakly and heterogeneously stained with HER2 during proliferative stage (Fig 2.15E), weak to medium during secretory stage (Fig 2.15F) and medium in post-menopausal glands (Fig 2.15G). Expression of HER2 on luminal epithelium was consistent during the menstrual cycle. Staining of glandular

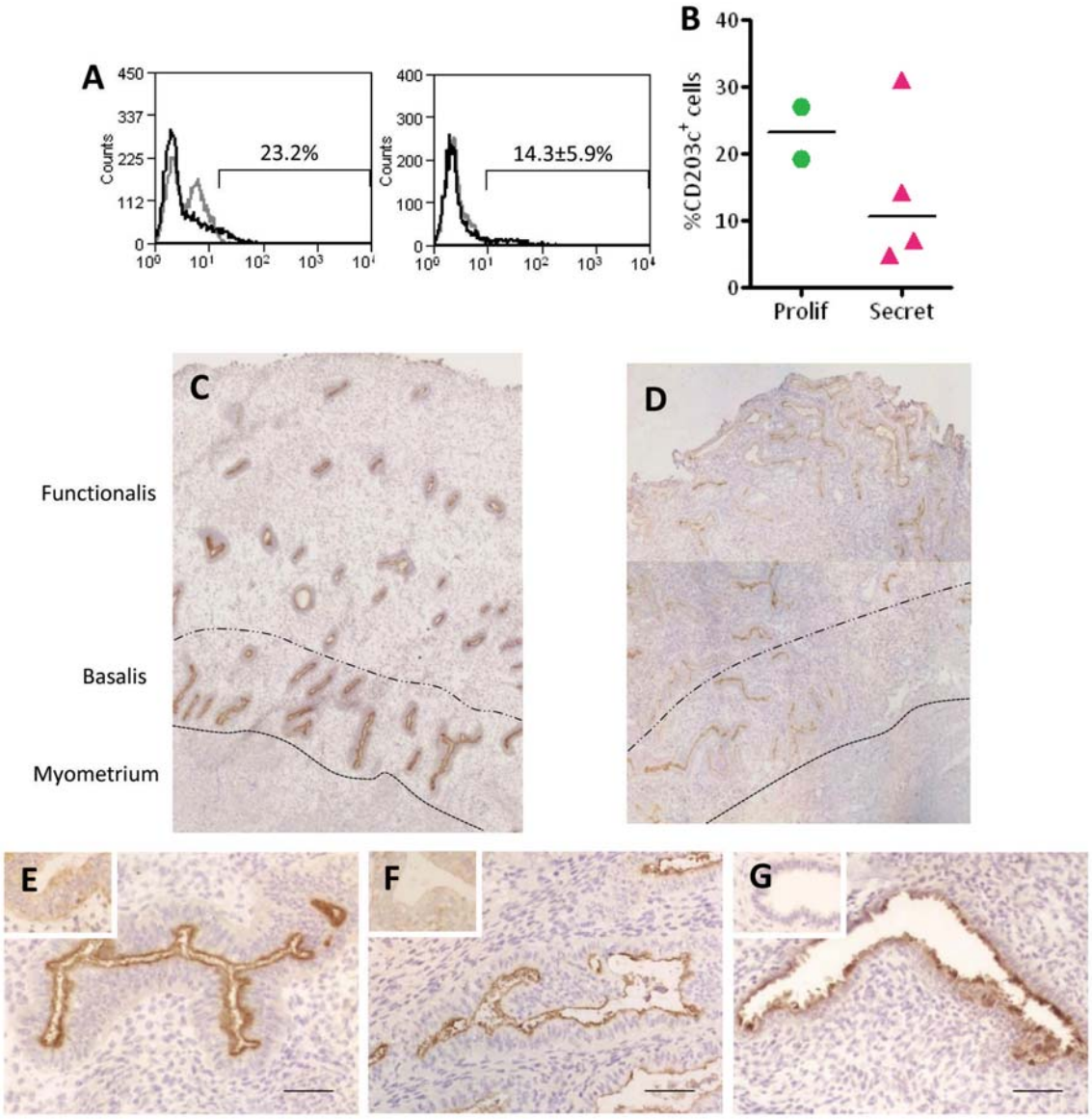


Figure 2.13: CD203c expression in human endometrium. (A) Representative single parameter flow cytometry histogram for CD203c, showing percentage of positive cells during proliferative (left, mean, n=2) and secretory (right, mean±SEM, n=4) stages. Black line: supernatant antibody. Grey line: isotype control. (B) Percentage of CD203c$^+$ epithelial cells in proliferative and secretory phase endometrial cell suspensions. Data is shown as a scatterplot, with each dot representing an individual sample. Solid bar represents median. CD203c staining of full thickness endometrial section at (C) proliferative and (D) secretory stages. 2.5X magnification. Representative staining of glandular epithelium during (E) proliferative, (F) secretory and (G) post-menopausal endometrium. Inset: representative section stained with isotype matched control. Scale bar: 100μm.

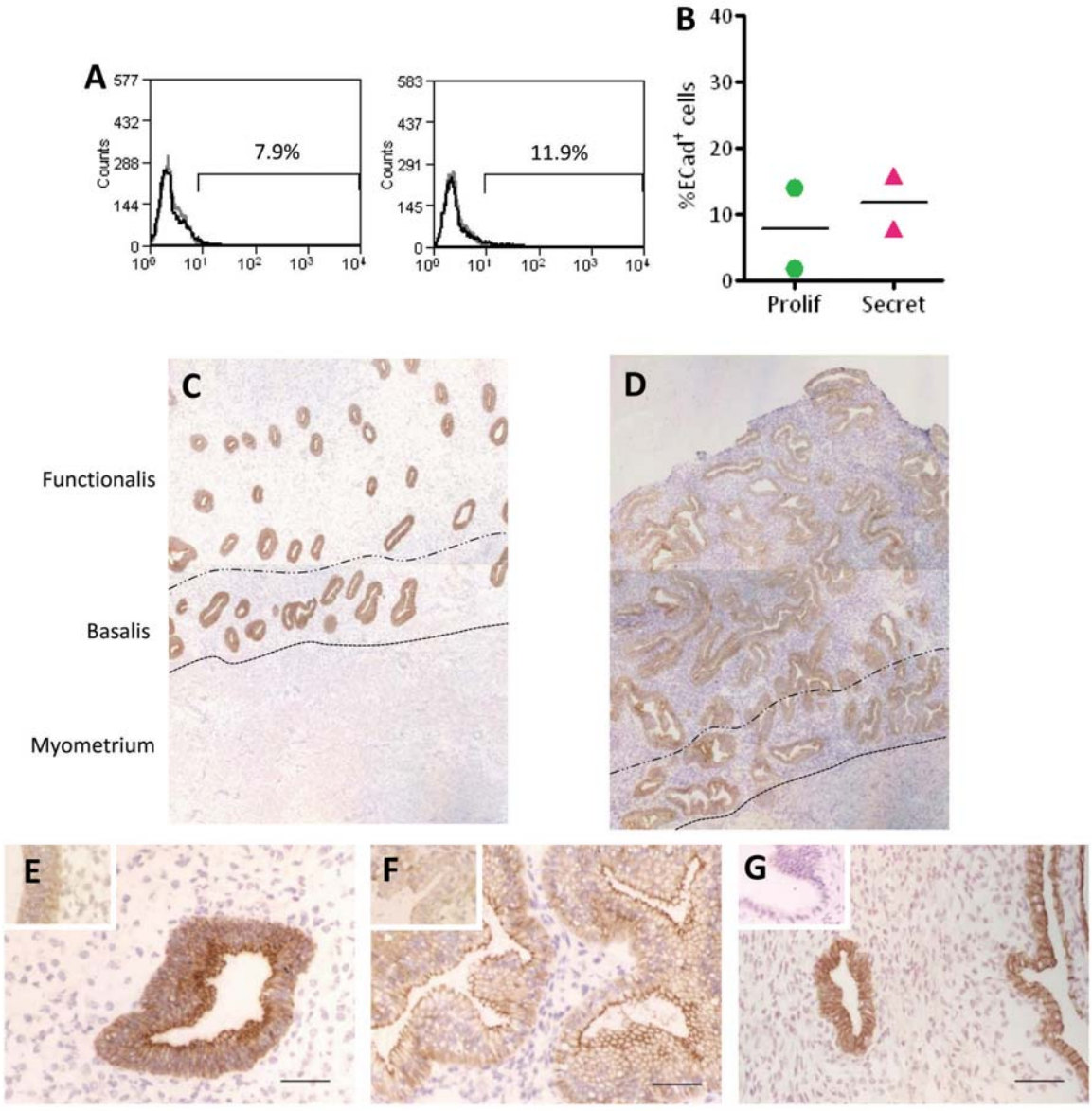


Figure 2.14: E-cadherin expression in human endometrium. (A) Representative single parameter flow cytometry histogram for E-cadherin showing percentage of positive cells during proliferative (left, n=2) and secretory (right, n=2) stages. Black line: supernatant antibody. Grey line: isotype control. E-cad, E-cadherin. (B) Percentage of E-cad $^+$ epithelial cells in proliferative and secretory phase endometrial cell suspensions. Data is shown as a scatterplot, with each dot representing an individual sample. Solid bar represents median. E-cadherin staining of full thickness endometrial section at (C) proliferative and (D) secretory stages. 2.5X magnification. Representative staining of glandular epithelium during (E) proliferative, (F) secretory and (G) post-menopausal endometrium. Inset: representative section stained with isotype matched control. Scale bar: 100μm.

epithelial cells was greater in the basalis compared to functionalis although overall staining was relatively weak (Fig 2.15C,D). HER2 was prioritised for its stronger expression on glandular epithelium in the basalis.

2.3.5.5 MSCA-1

MSCA-1, is expressed on bone marrow mesenchymal stem cells (Bühring et al., 2007) but had never been examined in human endometrium. This study found a small population of endometrial cells expressed MSCA-1 during proliferative (2.3%, n=2) and secretory (1.57%, n=1, Fig 2.16A,B) stage. There was no difference in staining intensity of glandular epithelial cells between the functionalis and basalis during proliferative (Fig 2.16C) and secretory stage (Fig 2.16D). Epithelial glands showed strong heterogeneous apical surface staining during proliferative stage (Fig 2.16E). The weak staining of epithelial glands during the secretory stage suggest that the epitope is down-regulated or luminally secreted (Fig 2.16F). MSCA-1 was not confined to epithelium and also stained perivascular cells (Fig 2.16G). Importantly, heterogeneous staining was observed in post-menopausal glands (Fig 2.16H), a finding that made MSCA-1 a priority.

2.3.5.6 HER3

HER3 (ErbB3), is expressed on epithelial cells (Ejskjaer et al., 2005) and has been associated with endometrial and pancreatic cancers (Srinivasan et al., 1999; Ejskjaer et al., 2007; te Velde et al., 2009). During the proliferative stage, expression was detected on 7.8% cells (n=1) and 13.3 $\pm$ 6.0% cells during the secretory stage (n=3, Fig 2.17A,B). The luminal epithelium and the basolateral surface of the glandular epithelium was weak to medium stained with HER3 antibody. Glandular epithelium was homogeneously stained during proliferative stage (Fig 2.17E) but interestingly was heterogeneous during secretory stage (Fig 2.17F). Expression of HER3 in glandular epithelia was stronger in the basalis compared to functionalis independent of the menstrual cycle (Fig 2.17C,D). And combined with the potential role of

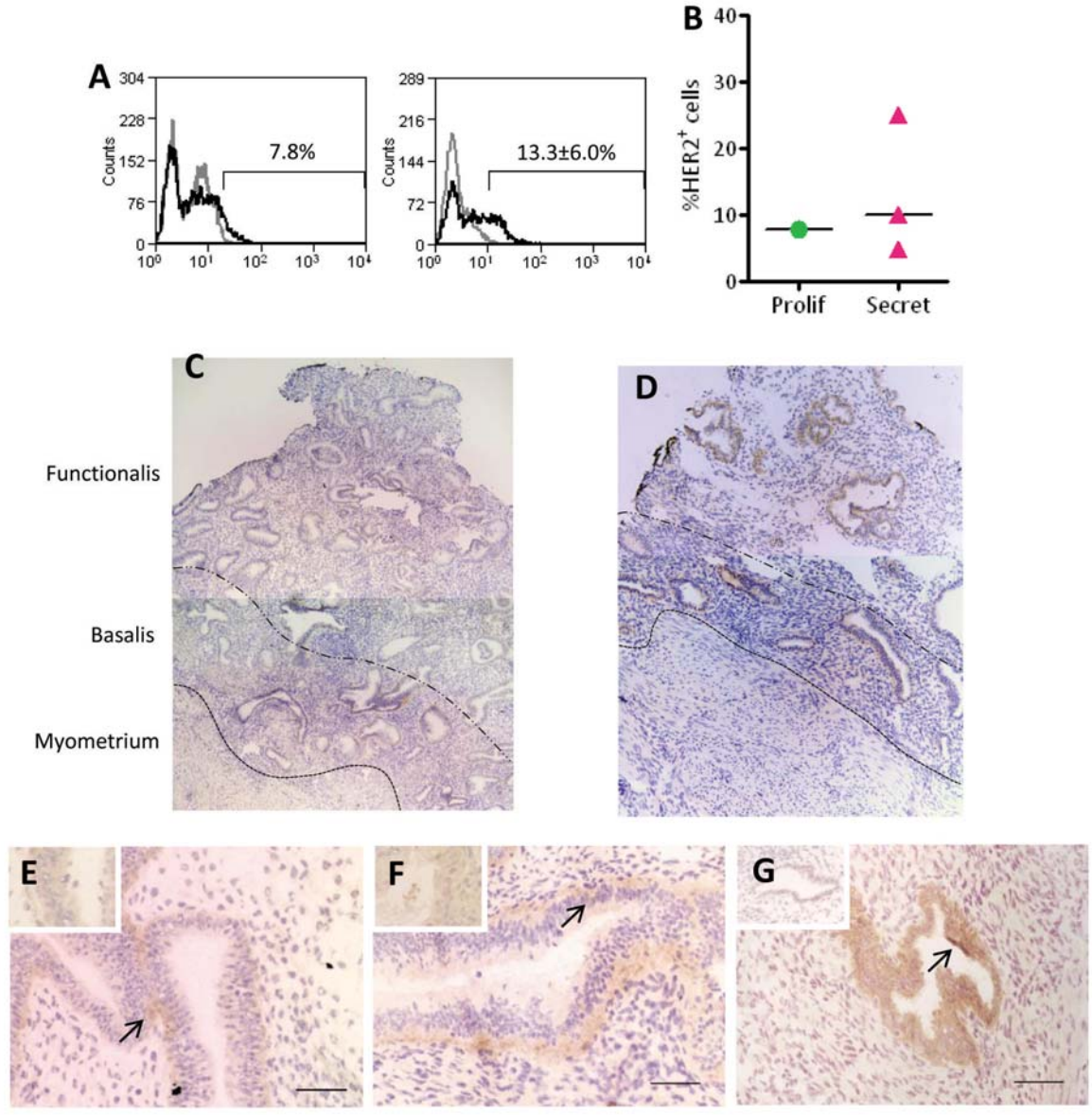


Figure 2.15: HER2 expression in human endometrium. (A) Representative single parameter flow cytometry histogram for HER2 showing percentage of positive cells during proliferative (left, n=1) and secretory (right, mean ± SEM, n=3) stages. Black line: supernatant antibody. Grey line: isotype control. (B) Percentage of HER2$^+$ epithelial cells in proliferative and secretory phase endometrial cell suspensions. Data is shown as a scatterplot, with each dot representing an individual sample. Solid bar represents median. HER2 staining of full thickness endometrial section at (C) proliferative and (D) secretory stages. 2.5X magnification. Representative staining of glandular epithelium during (E) proliferative, (F) secretory and (G) post-menopausal endometrium. Inset: representative section stained with isotype matched control. Heterogeneous staining (arrow). Scale bar: 100μm.

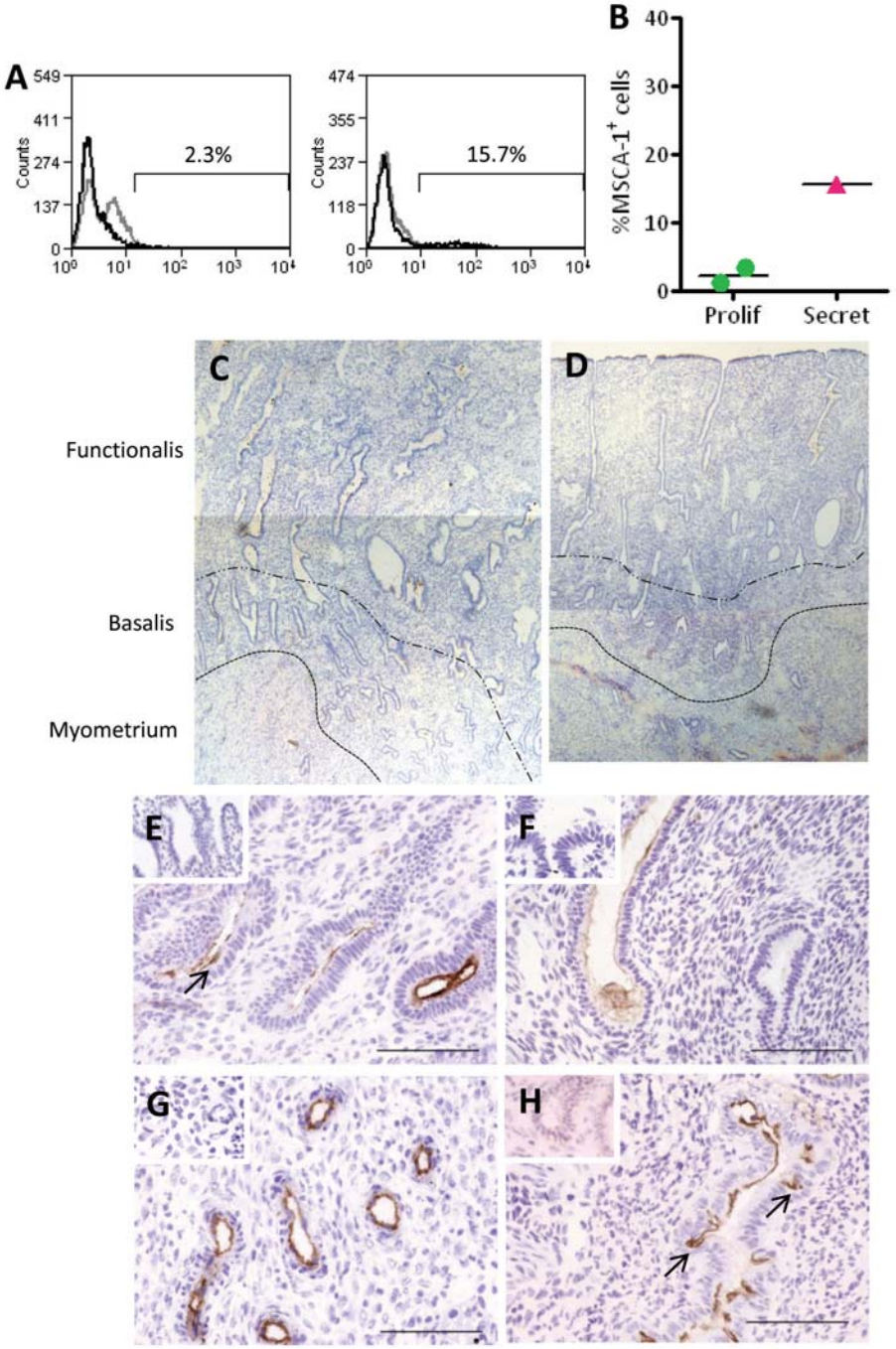


Figure 2.16: MSCA-1 expression in human endometrium. (A) Representative single parameter flow cytometry histogram for MSCA-1, showing mean percentage of positive cells during proliferative (left, n=2) and secretory (right, n=1) stages. Black line: supernatant antibody. Grey line: isotype control. (B) Percentage of MSCA-1 $^{+}$ epithelial cells in proliferative and secretory phase endometrial cell suspensions. Data is shown as a scatterplot, with each dot representing an individual sample. Solid bar represents median. Representative MSCA-1 staining of full thickness endometrial section at (C) proliferative and (D) secretory stages. 2.5X magnification. (E) Representative staining of glandular epithelium during proliferative, (F) secretory, (G) staining of a spiral ateriole and (H) post-menopausal endometrium. Inset: representative section stained with isotype matched control. Heterogeneous staining (arrow). Scale bar: 100μm.

HER3 in tumourgenesis increased its priority status for further investigation. Furthermore, the expression of HER3 on the basolateral surface of the glandular epithelium, indicated that it would not be secreted from the lumen and was thus a robust candidate marker.

2.4 Discussion

This study screened endometrial cells for immunoreactivity to a panel of novel and known supernatant antibodies to short list candidate epithelial progenitor cell markers. A majority of these markers had never been examined in the endometrium before. Screening was initially done by single-colour flow cytometry and those antibodies that were immunoreactive with endometrial cells were further pursued. Immunohistochemistry determined localisation of immunoreactivity in full thickness endometrium. Eighteen out of 24 antibodies did not immunoreact with the endometrium or did react to immunostain the stroma, perivascular cells and/or myometrium and were eliminated from the short list. Six out of the 24 (CD133, CD203c, E-cadherin, HER2, HER3 and MSCA-1) were specific for endometrial glandular epithelial cells and thus prioritised.

The short listing of candidate markers was not a straightforward task. Expression of antigens immunoreactive with each antibody was highly variable between the two major stages of the menstrual cycle. In addition, there was variation between patient samples, suggesting that hormones and growth factors that stimulate endometrial growth (Colville, 1968; Chabbert-Buffet et al., 1998; Salamonsen, 2006) may also influence marker expression. Another factor taken into consideration was epitope sensitivity to enzymatic digestion resulting in disconcordance between flow cytometry and immunohistochemistry findings, making the identification of a robust marker difficult.

To reduce the impact of experimental variability on determining the value of each marker for identifying endometrial epithelial progenitor cells, the expression of each marker was compared on (1) expression in epithelium (glandular or luminal localisation), (2) intensity

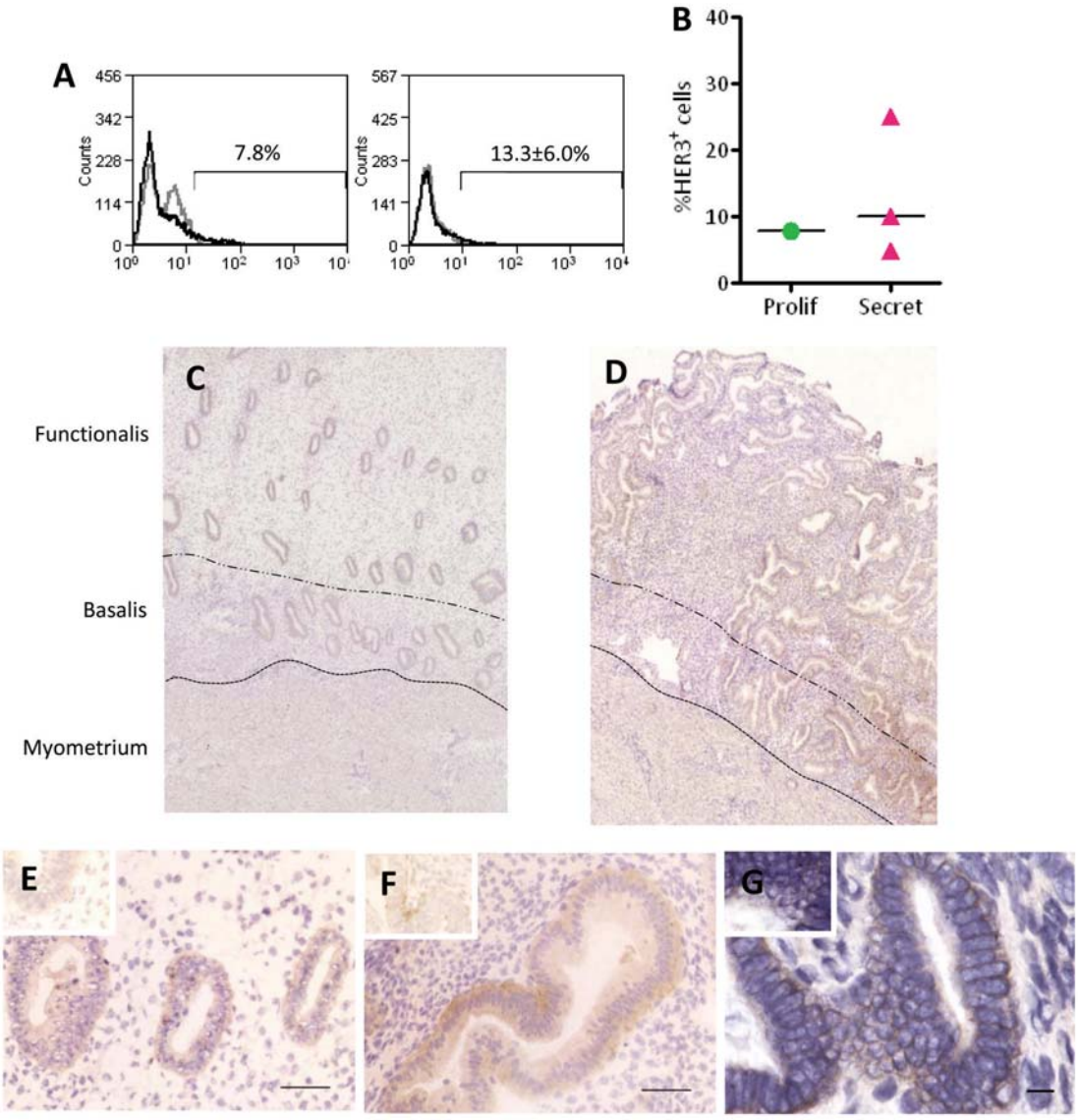


Figure 2.17: HER3 flow cytometry and immunohistochemistry summary. (A) Representative single parameter flow cytometry histogram for HER3, showing percentage of positive cells during proliferative (left, n=1) and secretory (right, n=3) stages. Black line: supernatant antibody. Grey line: isotype control. (B) Percentage of HER3 $^+$ epithelial cells in proliferative and secretory phase endometrial cell suspensions. Data is shown as a scatterplot, with each dot representing an individual sample. Solid bar represents median. Representative HER3 staining of full thickness endometrial section at (C) proliferative and (D) secretory stages. 2.5X magnification. Representative staining of glandular epithelium during (E) proliferative, (F) secretory and (G) post-menopause endometrium. Inset: representative section stained with isotype matched control. Scale bar: 100μm.

of glandular staining in the basalis compared to functionalis region, (3) immunostaining pattern of glandular epithelium (homogeneous or heterogeneous), (4) expression in inactive endometrium (present or absent) and (5) robustness to enzymatic digestion during tissue processing and would the antigen be secreted from the lumen. This strategy resulted in the prioritisation of six short listed candiate markers, HER3 being of precedence.

CD133 (AC133) was firstly identified on human hematopoietic stem and progenitor cells (Miraglia et al., 1997) and has been exclusively located on microvili (Weigmann et al., 1997; Karbanova et al., 2008). CD133 has been used as a prospective marker for the isolation of stem and cancer stem cells in blood, brain, prostate and colon (Yin et al., 1997; Uchida et al., 2000; Richardson et al., 2004; Marzesco et al., 2005; O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Vander Griend et al., 2008). In the human endometrium, CD133 was the first marker used to identify and prospectively isolate endometrial carcinoma (EC) stem cells that were highly proliferative and more resistant to the toxic effects of chemotherapy agents (Rutella et al., 2009). In the normal endometrium, this study found immunoexpression was specific for luminal and glandular epithelium, in agreement with previous reports (Karbanova et al., 2008; Schwab et al., 2008). Expression of CD133 appeared to be down-regulated in secretory stage epithelium, which could account for negative immunohistochemistry results reported previously (Miraglia et al., 1997). However, the strong intensity of immunostaining observed during proliferative stage was not reflected by flow cytometry analysis. This discrepancy could be due to the negative effects of mechnical and proteolytic digestion on surface markers. Previously, flow cytometry used to examine the effects of enzymatic digestion upon peripheral blood T lymphocyte surface markers found a marked decrease in surface marker expression (Abuzakouk et al., 1996). These enzyme-induced cell surface alterations could have also affected receptors involved with viability and proliferation. These reasons could also account for the observation that freshly Collagenase 1/Typsin-EDTA dissociated $CD133^{+}$ EC cells were not capable of forming xenograft tumors (Rutella et al., 2009). In addition, CD133 was detected in a variety of adult tissues by RT-PCR however expression could not be

detected by immunohistochemistry (Corbeil et al., 2001). An alternative explanation for the discrepancy between flow cytometry and immunostaining data is the CD133 epitope depends upon glycosylation thus can only be detected at certain stages of cellular differentiation (Corbeil et al., 2001), indicating that CD133 would not be a robust candidate marker of endometrial epithelial progenitors. Nevertheless, given the reported success of CD133 as a prospective marker of stem/progenitor cells, it was short listed.

CD203c recognizes basophils and mast cells (Bühring et al., 2001) and is involved in allergies (Hauswirth et al., 2002). CD203c has been suggested as a serum biomarker of colon and bile duct carcinoma (Yano et al., 2003, 2004). In the human endometrium, CD203c is highly expressed by luminal and glandular epithelia, in agreement with previous observations albeit limited (Bühring et al., 2004). CD203c was shortlisted as a potential marker because of its stronger basalis compared to functionalis expression, despite the observation that most epithelial cells expressed this antigen. However, since it is an apical membrane marker it could potentially be vulnerable to proteolytic digestion (Abuzakouk et al., 1996) or secreted into the lumen (Bühring et al., 2004), and thus was not top priority for this study. However in future, CD203c could be used as a marker for the isolation of basalis epithelial cells, the region where the endometrial stem/progenitor niche is expected. Or could be used to examine endometriotic lesions to determine if they contain epithelial cells from the basalis and thus possibly support the theory of incorrectly shed basalis as a cause of endometriosis.

E-cadherin is specific for endometrial epithelial cells (Beliard et al., 1997; Poncelet et al., 2002), a finding concurrent with this study. E-cadherin is a molecule that contributes to the stability of intercellular adhesion between all epithelial cells (Gumbiner, 1996). When E-cadherin$^-$ ESC lines were injected into nude mice, tumours formed were devoid of any organized structures (Larue et al., 1996). In contrast, E-cadherin expressing ESC lines formed only epithelia (Larue et al., 1996) emphasizing the importance of E-cadherin in tissue formation in the highly regenerative endometrium. Interestingly, there was greater expression of E-cadherin in the basalis compared to functionalis thus short listing this candidate marker.

However flow cytometry and immunohistochemistry results did not correlate. This suggested that epitopes were enzyme sensitive during tissue digestion and subsequently E-cadherin was ranked lower. Perhaps the increased basalis expression ensures the integrity of this section during menstruation and reduced E-cadherin expression may lead to basalis being sloughed off and may lead to endometriosis.

In the endometrium, the recently described MSC marker MSCA-1, stained epithelial glands as well as perivascular cells. During this screening study, this novel marker had not yet been fully characterized and was referred to by its clone name W8B2B10 as it's specificity was unknown (Bühring et al., 2007). However during screening of W8B2 on endometrial cells, the novel antigen was reported to prospectively isolate bone marrow derived mesenchymal stem cells and was designated MSCA-1 (Battula et al., 2009). Further studies showed that MSCA-1 was identified as TNAP (tissue non-specific alkaline phosphatase), an ectoenzyme highly expressed in liver, bone and kidney as well as embryonic stem cells (Sobiesiak et al., 2010). As embryonic stem cells differentiate, MSCA-1 decreases (Sobiesiak et al., 2010), suggesting that MSCA-1 is a marker of primitive stem cell-like cells, and would be of more interest for this screening study. In the endometrium, MSCA-1 was localised on perivascular cells which interestingly is where mesenchymal stem cell-like cells are shown to reside (Schwab and Gargett, 2007). MSCA-1 is also co-expressed by $CD146^{+}$ cells (Sobiesiak et al., 2010), the latter a published marker of mesenchymal stem cell-like cells from the endometrium and other tissues (Shi and Gronthos, 2003; Schwab and Gargett, 2007; Schwab et al., 2008). Without this knowledge, MSCA-1 was short listed because of its stronger glandular staining during proliferative compared to secretory stage endometrium. This is in accord with early studies assessing alkaine phosophatase activity in the endometrium (McKay et al., 1956). In addition, this study also found heterogeneous glandular staining in post-menopausal endometrium implying that MSCA-1 may isolate a subset of glandular epithelial cells that persist throughout a lifetime and could potentially mark an epithelial progenitor population.

HER2 and HER3 belong to the ErbB family of receptors that trigger signaling pathways

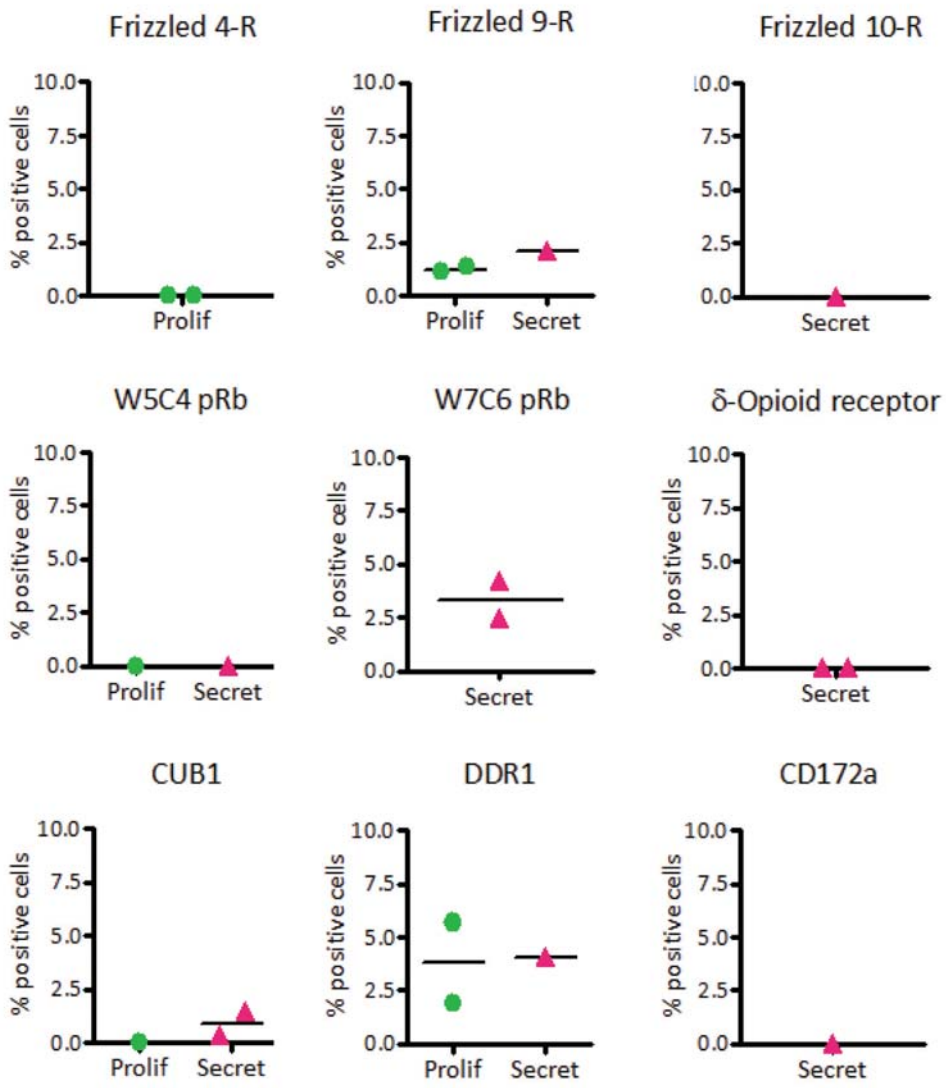
resulting in cell division, death, motility and adhesion (Yarden and Sliwkowski, 2001). Weak expression of HER2 has been reported in normal endometrium (Rasty et al., 1998) supporting observations made in this study. There was greater HER2 expression in the secretory stage and stronger expression in glandular epithelium of the basalis compared to functionalis. For these reasons and because the overexpressed HER2 is in epithelial tumours, it was assigned priority (Slamon et al., 1987; Rasty et al., 1998; Punnoose et al., 2010). Low levels of HER3 expression have been reported in normal endometrium, with stroma showing some weak expression (Srinivasan et al., 1999). Low levels of HER3 were also observed in this study however plasma membrane immunoreactivity was localised specifically to the glands. This discrepancy might be due to methodological differences particulary the antibody used because this study used clone D1D12 rather than RT.J2. This study found greater HER3 expression during secretory stage compared to proliferative. There was also consistently greater endometrial expression in basal epithelial glands compared to functionalis at different stages of the menstrual cycle and is of particular interest given the endometrial stem cell niche is hypothesised to be located in the basalis. Interestingly, the intensity of immunostaining in post-menopausal endometrium was comparable to proliferative stage endometrium, suggesting that HER3 expressing cells persist in endometrial tissue for a lifetime, and could be present on epithelial progenitors.

There were a few limitations of this screening study. Firstly, in terms of flow cytometry there were not enough cells in individual patient samples to test all antibodies in a single experiment thus reported values show high patient variability. Secondly, the stage of menstrual cycle could not be selected in advance as samples needed to be processed immediately before pathology reports were available. This meant that some antibodies were tested on more secretory samples than proliferative samples and vice versa. Thirdly, fewer hysterectomies were available as the project progressed due to increased medical management of gynaecological problems, thus reducing the number of samples available for experiments. Also, the small size of tissue samples able to be collected meant that an individual patient sample had to be devoted in its entirety for either flow cyometry or immunohistochemistry. Curette samples

were more readily obtained. However, curettage samples the functionalis with relatively little basalis endometrium retrieved (Skinner et al., 1999). Since the endometrial stem cell niche is hypothesised to be basally located, curettings were not a suitable source of tissue samples and kept to a minimum. Where curettings were used for antibody optimisation, optimisation was repeated on a full thickness endometrial sample. Finally, dead cells, erythrocytes and leukocytes were not removed from the cell suspension. It is possible that these cells could have contributed to a percentage of cells reacting with antibodies during incubations for flow cyometry analysis although the forward and side scatter gating should have removed most due to their small size, a more vigorous analysis would have required more time and samples and would have hindered the progress of this study. Despite these limitations, the current strategy yielded six candidate markers. The addition of electron microscopy could have also been used to enhance to visualisation of the location of antigen epitopes in endometrium. Nonetheless, the screening by flow cytometry and immunohistochemistry reported in this chapter was sufficient to determine endometrial reactivity and fulfilled the aim of generating a priority list.

In summary, prioritization and higher ranking of a prospective marker, was assigned on the basis of greater basalis expression where stem cells are hypothesised to reside, robustness against enzymatic digestion and cyclical hormonal variations and expression in postmenopausal endometrial epithelial cells suggesting permanency in the inactive endometrium. Six markers made this priority short list of candidate epithelial progenitor markers. The top ranked, HER3 is further examined in the next chapter.

2.5 Supplementary Information



Supplementary Figure 2.18: Flow cytometry results of antibodies that were not prioritised.

CHAPTER 3

HER3 is a putative marker of human endometrial epithelial progenitor cells

3.1 Introduction

During the reproductive life of a woman, the endometrium regenerates over 400 times following menstruation in the absence of a pregnancy. Stem cells have been postulated to be responsible for this remarkable rapid cellular proliferation (Padykula, 1991; Gargett, 2007) and may also have a role in the aetiology of gynaecological disorders (Gargett and Masuda, 2010). Since the first evidence of epithelial progenitor cells from the human endometrium was reported (Chan et al., 2004; Schwab et al., 2005) efforts have been made to further characterise this rare population. These studies demonstrate epithelial progenitor cells as clonogenic, self-renewing and able to differentiate into gland-like structures *in vitro* (Chan et al., 2004; Schwab et al., 2005; Gargett et al., 2009). Other studies have shown that stemness genes are expressed in the human endometrium although this has not been investigated in epithelial progenitor cell populations (Forte et al., 2009; Bentz et al., 2010). Nonetheless, the most convincing evidence to date demonstrates endometrial single cell suspensions (including epithelial and stromal cells) as capable of reconstituting epithelial glands in xenotransplantation assays (Masuda

et al., 2007). Whilst these data contribute valuable knowledge of human endometrial epithelial progenitor cell activity, the assays used are retrospective and do not allow the identification of epithelial progenitor cells. The lack of a marker that prospectively isolates endometrial epithelial progenitor cells hampers their further characterisation.

Attempts to prospectively identify human endometrial epithelial progenitor cells have used flow cytometry sorting for side-population (SP) cells (Kato et al., 2007; Tsuji et al., 2008; Cervello et al., 2010; Masuda et al., 2010). Initally identified in bone marrow (Goodell et al., 1996), the SP technique relies upon the expression of *Brcp1/ABCG2* that confers the ability of stem cells to efflux the Hoechst 33342 fluorescent dye (Goodell et al., 1996, 1997; Zhou et al., 2001). Studies of the haemopoietic system revealed Brcp1/ABCG2^{+} SP cells represented a heterogeneous population of stem and lineage-committed cells (Zhou et al., 2002; Naylor et al., 2005), suggesting that the expression of *Brcp1/ABCG2* may not necessarily confer stemness. Despite this, endometrial SP cells have differentiated to form organised structures *in vitro* and in xenografts as well as demonstrating stem cell-like activity (Kato et al., 2007; Tsuji et al., 2008; Cervello et al., 2010; Masuda et al., 2010). However, it is a heterogeneous population comprising predominantly of endothelial cells but also epithelial and stromal cells (Tsuji et al., 2008; Cervello et al., 2010; Masuda et al., 2010), thus the identification of a marker that prospectively isolates purified epithelial progenitor cells is imperative.

In an effort to identify a prospective marker for the prospective isolation of epithelial progenitor cells, a panel of novel and known antibodies was screened for immunoreactivity to the endometrium (Chapter 2). HER3 was identified as a candidate marker. HER3 (ErbB3) belongs to the ErbB family of receptors involved in development and differentiation in a number of tissue systems including cardiac, nervous and mammary (Casalini et al., 2004). This family of receptors has also been investigated for their role in the pathogenesis of endometrial and mammary cancers (Rasty et al., 1998; Ejskjaer et al., 2007; Hsieh and Moasser, 2007; Campbell et al., 2010; Koutras et al., 2010). In normal endometrium, HER3 localised specifically to epithelial glands during all stages of the menstrual cycle (Chapter 2)

(Prigent et al., 1992; Ejskjaer et al., 2005). Expression of HER3 was greater in the basalis region compared to functionalis (Chapter 2), the former where the endometrial epithelial progenitor niche is hypothesized to reside (Gargett, 2007). Interestingly, HER3 was detected in the inactive endometrium (Chapter 2), suggesting these HER3$^+$ cells can persist throughout a lifetime independent of hormonal influence.

The aim of this study was to assess HER3 as a potential marker for the prospective isolation of clonogenic human endometrial epithelial cells. To achieve this, fresh human endometrial epithelial cells were cell sorted into subpopulations based upon the relative expressions of HER3 and EpCAM (epithelial marker) using flow cytometry. Colony-forming and self-renewal assays and expression of pluripotent and self-renewal genes were investigated to determine which subpopulation was enriched for endometrial epithelial progenitor cells.

3.2 Materials & Methods

3.2.1 Human Tissues

Endometrial tissues (n=30, Table 3.1 and Suppl Table 3.14) were collected from ovulating women aged (44.1 $\pm$ 0.9 years, range 31–51) undergoing hysterectomy (n=27) or curettage (n=3). Women had not received hormonal treatment three months prior to surgery. 12 patients were in the proliferative menstrual phase, 14 in secretory, two were menstruating, two had poorly developed endometrium. Full thickness endometria with 5 mm attached myometrium or curettes were collected in Collection Medium (DMEM/F12 with HEPES; 1% Antibiotics, both Invitrogen; 2% fetal calf serum, Gibco/Invitrogen) and processed within 2–24 hr, or frozen in OCT Tissue Tek (Sakura Finetek Co., Tokyo, Japan) on dry ice and stored at -80 °C until required.

Ethics approval was obtained from the Monash Medical Centre Human Research and Ethics Committee B. Informed written consent was obtained from each patient. The stage of

Sample	Age	Menstrual Stage	Pathology
13-09	36	P	Leiomyomata
15-09	46	S	Menorrhagia
20-09	42	MS	Menorrhagia
21-09	41	P	Prolapse
37-09	47	EP	Menorrhagia
42-09	35	LS	CUR, infertility
43-09	34	P	CUR, infertility
47-09	42	P	Prolapse
68-09	45	P	Menorrhagia
112-09	45	Poorly developed S	Leiomyomata
119-09	46	MS	Menorrhagia
03-10	45	P	Menorrhagia
43-10	49	P	Pain
53-10	37	LS	Prolapse
84-10	41	MS	Menorrhagia
90-10	45	LS	Prolapse
115-10	45	LS	Prolapse
158-10	38	P	CUR, infertility
165-10	49	S	Leiomyomata
175-10	51	P	Menorrhagia
195-10	49	LS	Menorrhagia

Table 3.1: Patient sample characteristics. Menstrual stages: early proliferative (EP), proliferative (P), secretory (S), mid-secretory (MS), late secretory (LS). Pathology: curette (CUR), all other samples are hysterectomies

the menstrual cycle of the samples was assessed by histological examination of hematoxylin and eosin stained sections according to experienced pathologists using well established criteria for the normal menstrual cycle (Noyes et al., 1975) and was obtained from pathology reports. Post-menopause (inactive endometrium) was defined by $\geq$12 consecutive months of no menstruation (amenorrhea).

3.2.2 Preparation of single cell suspensions of human endometrial cells

Human endometrium was dissociated to a single cell suspension as described in Chapter 2 with minor modifications. Briefly, tissue was mechanically dissociated using scalpels followed by

enzymatic digestion in Collagenease type 1 (5mg/ml; Worthington Biochemical Corporation, New Jersey, USA)/PBS and DNase1 (0.5mg/ml; Worthington Biochemical Corporation)/PBS for 1.5 hr followed by Collagenase type 2 (4mg/ml; Worthington Biochemical Corp)/PBS and DNase1 (1mg/ml; Worthington Biochemical Corporation)/PBS for 30–40 min (Fig 3.2).

3.2.3 Flow cytometry sorting with HER3 and EpCAM

Freshly isolated endometrial epithelial cell suspensions (5x10^4–8x10^6 cells) were blocked with rat serum (5%, Sigma, St. Louis, USA) in Flow Buffer (100μl, 2% fetal calf serum (FCS; Invitrogen)/PBS) and incubated with antibodies against HER3 (250μl/ml dilution of supernatant as optimised in Chapter 2; clone D1D12; mouse IgG_1; Dr Hans-Jörg Bühring, Tübingen, Germany) and biotinylated goat anti-human EpCAM (5μg/ml; IgG; R&D Systems, Minneapolis, MN, USA). The endometrial epithelial cancer cell line (ECC-1) was used as a positive control (Suppl 3.5.2) and mouse IgG_1 isotype antibody (Dako Cytomation, Glostrup, Denmark) or biotinylated goat IgG_1 (5μg/ml; R&D Systems) as a negative controls for 30 min at 4 °C. Subsequently, cells were blocked with rat serum and incubated with PE-conjugated rat anti-mouse IgG_1 (1μg/ml; BD Pharmingen, San Diego, CA, USA) and streptavidin APC-A750 (1μl per 20x10^6 cells; Caltag/Invitrogen) for 20 min at 4 °C. Cells were further incubated with A647-conjugated anti-CD31 (5μg/ml; BD Pharmingen), APC-conjugated anti-CD45 (2.5μl per 1x10^6 cells; Caltag/Invitrogen) and APC-conjugated anti-CD90 (5μg/ml; BD Pharmingen) for 20 min at 4 °C, and resuspened in Flow Buffer containing 7-Aminoactinomycin D (7AAD, 1μg/ml, Sigma-Aldrich) or SYTOX Blue (1μM; Molecular Probes Invitrogen; Suppl 3.5.3 for optimisation) and taken for immediate flow cytometry sorting using a MoFlo flow cytometer (DakoCytomation, Fort Collins, CO, USA) and Cyclops SUMMIT software (Version 5.2; DakoCytomation). Cells were selected for analysis by electronically gating the forward versus side scatterplot (Fig 3.3A), for single cells (Fig 3.3B), absence of CD31 (endothelial), CD45 (erythrocyte) and CD90 (stromal) expression (Fig

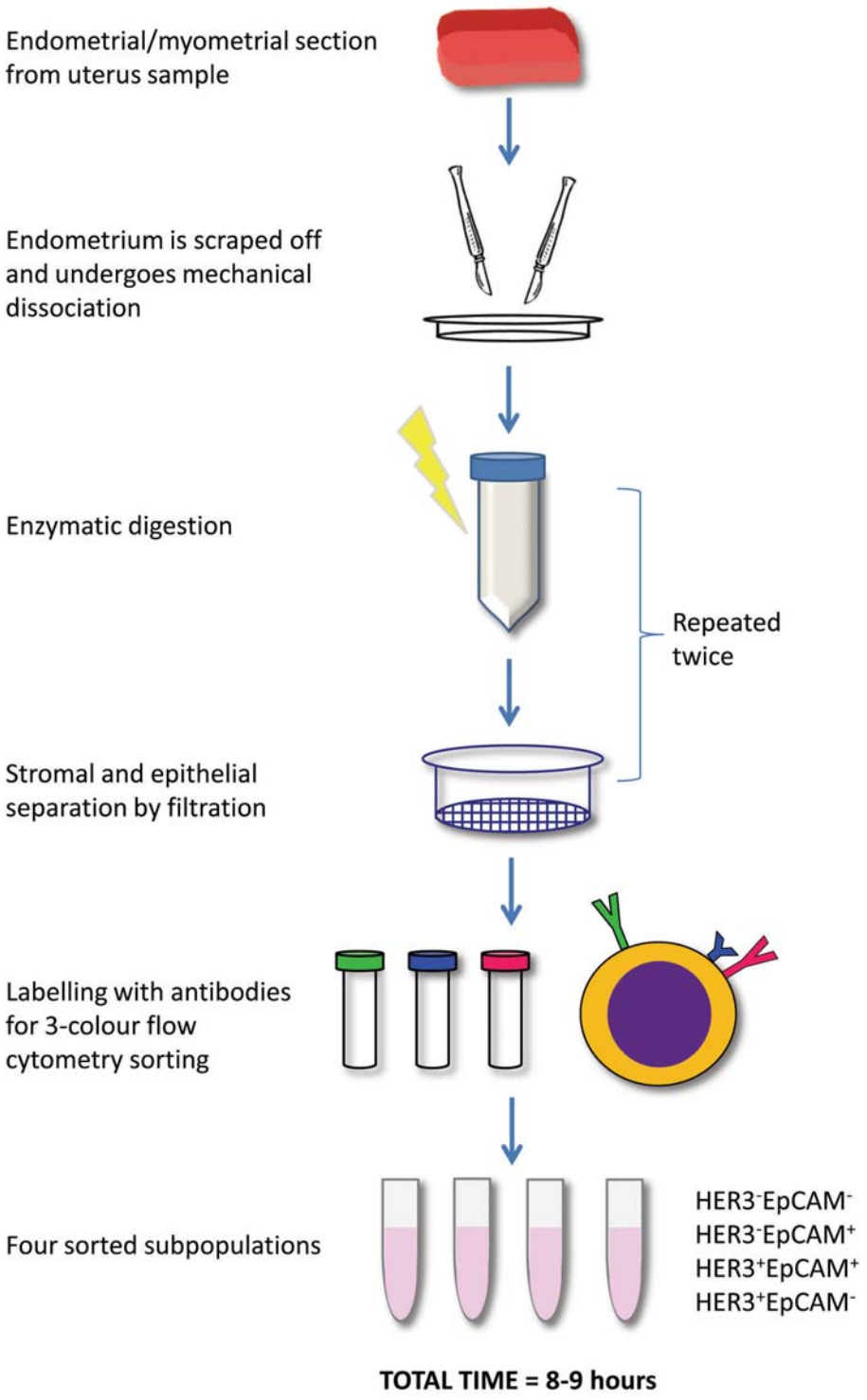


Figure 3.2: Protocol for the isolation of human endometrial epithelial cell suspensions for flow cytometry sorting.

3.3C), and viability (7AAD$^-$ or SYTOX Blue$^-$; Fig 3.3D). A minimum of 10,000 events from the isotype controls were used to determine electronic gating for negative cells (Fig 3.3A). Selected cells were sorted into two subpopulations on the basis of HER3 expression (HER3$^+$ and HER3$^-$ cells, Fig 3.7) or four subpopulations based upon the relative level of HER3 and EpCAM expression (HER3$^+$EpCAM$^-$, HER3$^+$EpCAM$^+$, HER3$^-$EpCAM$^+$ and HER3$^-$EpCAM$^-$, Fig 3.3E) into tubes containing Flow Collection Medium (DMEM/F12, 20% FCS, 1% antibiotics) at 4 °C. To determine if flow cytometry sorting affected cell viability and proliferation, sorted viable cells (7AAD$^-$ or SytoxBlue$^-$) and an aliquot of non-sorted cells were also collected as experimental controls.

3.2.4 Preparation of stromal feeder layers

Fresh stromal cells isolated and cultured from a hysterectomy sample were passaged as a feeder layer, using the Mitomycin-C inactivation and seeding density initially optimised using an immortalised endometrial stromal cell line. With the intention of xenotransplanting stromal cells with sorted HER3/EpCAM subpopulations in future, it was thought best that an immortalised endometrial stromal cell line should not be co-transplanted amid concerns of potential rapid stromal cell overgrowth. Fresh stromal cells from a single hysterectomy sample (#58-10) were used for the purpose of a feeder layer and cell isolation was undertaken as described previously (Section 3.2.2). An alternative was to use Briefly, endometrium was mechanically disaggregated and tissues were further dissociated in Collagenase type 1 for 1.5 hr and filtered to remove clusters of glandular epithelium. Erythrocytes and dead cells were removed from the filtrate by a Ficoll-Paque density gradient centrifugation method (Section 3.2.2). Stromal cells were seeded at 10,000 cells/cm^2 and cultured for 3–4 days or until 70–80% confluent. Cells were washed with PBS and inactivated with Mitomycin-C (10μg/ml; Sigma-Aldrich)/bicarbonate-buffered DMEM/F12 medium containing 10% FCS, 2mM glutamine (Invitrogen) and antibiotic-antimycotic for 2 hr at 37 °C in 5% CO_2. After

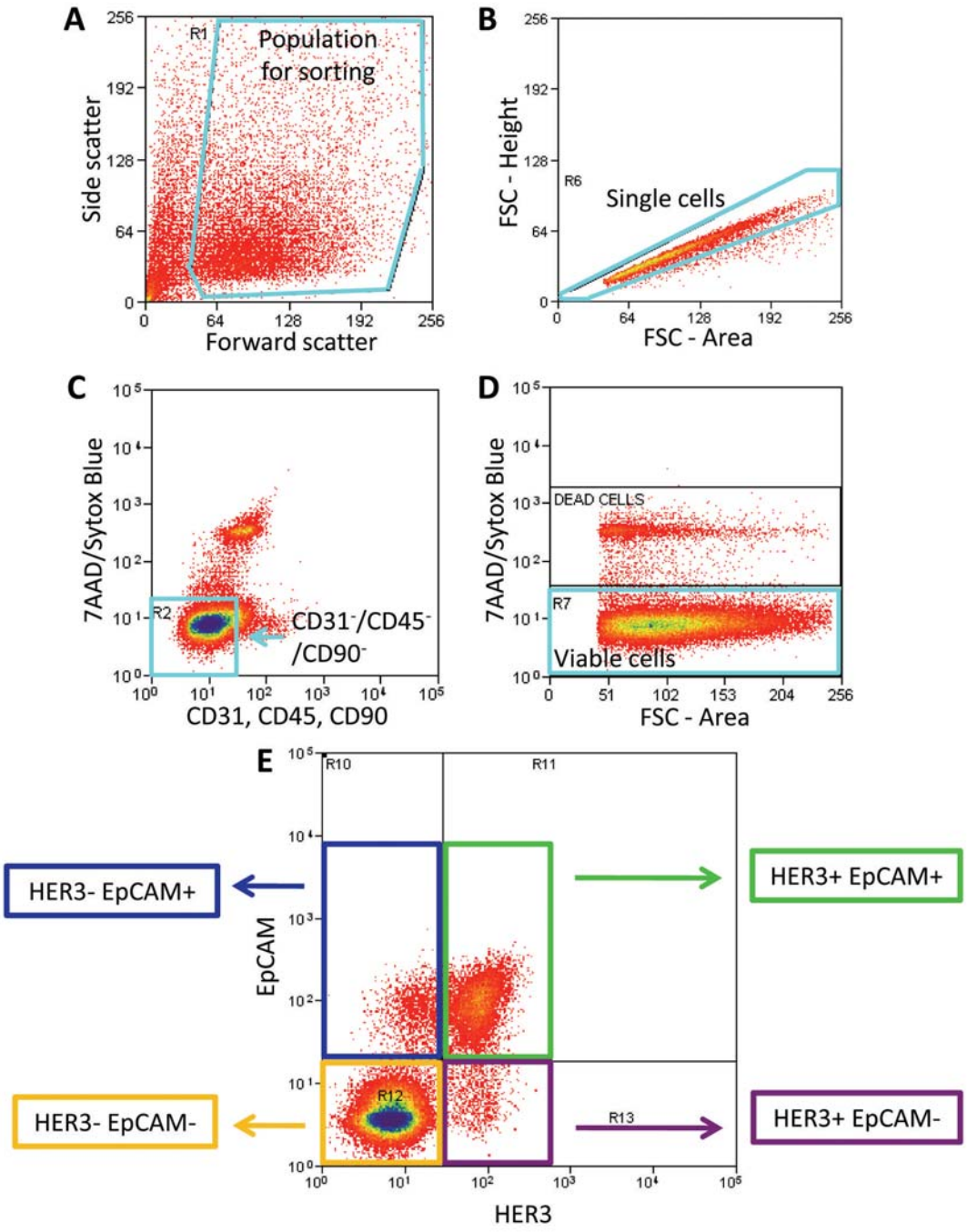


Figure 3.3: Electronic gating strategy for flow cytometry sorting of endometrial epithelial cells. (A) The population for sorting is selected through electronic gating (outlined in light blue). (B) Only single cells have been included. (C) Endothelial ($CD31^+$), erythrocytes ($CD45^+$) and stromal cells ($CD90^+$) were excluded. (D) Only viable cells were sorted ($7AAD^-$/Sytox $Blue^-$. (E) Finally cells are sorted into four subpopulations based upon relative expression of HER3 and EpCAM. FCS, forward scatter.

Mitomycin-C treatment, cells were washed with PBS and removed from the flask using Tryple Express (Invitrogen), seeded at 3,000 cells/cm^2 (Supplementary 3.5.4 for optimisation) onto fibronectin (BD Biosciences)-coated 60 or 100 mm Petri dishes (BD Discovery Labware, Bedford, MA, USA), maintained at 37 °C in 5% CO_2 and used within 1–5 days post inactivation.

3.2.5 *In vitro* colony-forming unit assay to assess clonogenicity

Initially, flow cytometry sorted HER3$^+$ and HER3$^-$ cells did not form any colonies when cultured on fibronectin coated plastic. Subsequently, culture methods were modified so that the four cell sorted subpopulations of HER3 and EpCAM labelled cells and experimental controls (sorted viable cells (7AAD$^-$/SytoxBlue$^-$) and non-sorted cells) were co-cultured with Mitomycin-C inactivated stromal feeder layers. All sorted subpopulations were seeded at two clonal densities (100 cells/cm^2 and 200 cells/cm^2) in duplicates into fibronection (BD Biosciences)-coated 60 or 100mm Petri dishes in bicarbonate-buffered DMEM/F12 medium containing 10% FCS, 10ng/ml Epidermal Growth Factor (human recombinant; BD Biosciences), 2mM glutamine and Primocin (100μg/ml; Invivogen, San Diego, CA, USA) at 37 °C in 5% CO_2. Media was changed every 6–7 days and colonies were monitored every 2–3 days to ensure that they were derived from single cells. Cultures were terminated at 14–30 days, fixed in 10% Formalin (Amber Scientific, Midvale, Australia)/PBS and stained with Harris Hematoxylin Solution (Amber Scientific). Colony-forming units (CFU) $\geq$50 cells were counted on $\geq$three plates at the different clonal densities and averaged (Chan et al., 2004; Schwab and Gargett, 2007; Gargett et al., 2009; Hubbard et al., 2009). Colony-forming efficiency (CE%) = [(number of CFU) / (number of cells seeded) x 100] was determined (Chan et al., 2004; Schwab and Gargett, 2007; Gargett et al., 2009; Hubbard et al., 2009).

3.2.6 Subcloning of *in vitro* colony-forming units to assess self-renewal

Individual CFU cultured from each of the four flow cytometry sorted subpopulations and experimental controls (sorted viable cell-derived CFU and unsorted cell-derived CFU) were subcloned after 14–30 days in culture (Fig 3.4; Gargett 2009; Hubbard 2010). From each sorted subpopulation, $\geq$three individual large primary clones ($\geq$800 cells each) were selected for subcloning. Well separated individual clones were incubated with Tryple Express (Invitrogen) in cloning rings (Sigma-Aldrich) and replated at 10–60 cells/cm^2 to generate secondary clones (Gargett et al., 2009). Following another four weeks in culture, a single secondary clone generated from each primary clone was re-cloned to generate tertiary clones (Fig 3.4). After another four weeks in culture, plates were fixed and stained as described (Section 3.2.5).

3.2.7 Immunohistochemistry

Immunohistochemistry was carried out as previously described (Section 2.2.4). Briefly, supernatant HER3 antibody supernatant was used at 250μl/ml dilution. Bench medium (25μl; DMEM/F12 with HEPES, 10% Newborn Calf Serum, 1% antibiotics; Invitrogen) was added to the isotype control dilutent (2.5μl; mouse anti-human IgG$_1$; Dako Cytomation) 0.1% BSA/PBS to recreate a supernatant effect. EpCAM, an epithelial marker (2.5μg/ml; mouse anti-human IgG$_1$; clone Ber-EP4; Dako Cytomation) was used as a positive control.

3.2.8 RNA isolation and PCR

Sorted HER3/EpCAM subpopulations were assessed for expression of pluripotent and self-renewal genes using reverse transcription - polymerase chain reaction (RT-PCR). RNA was isolated and genomic DNA contamination was removed using RNAqueous Kit (Ambion, Austin, TX, USA) according to manufacturer instructions. Total RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Counts

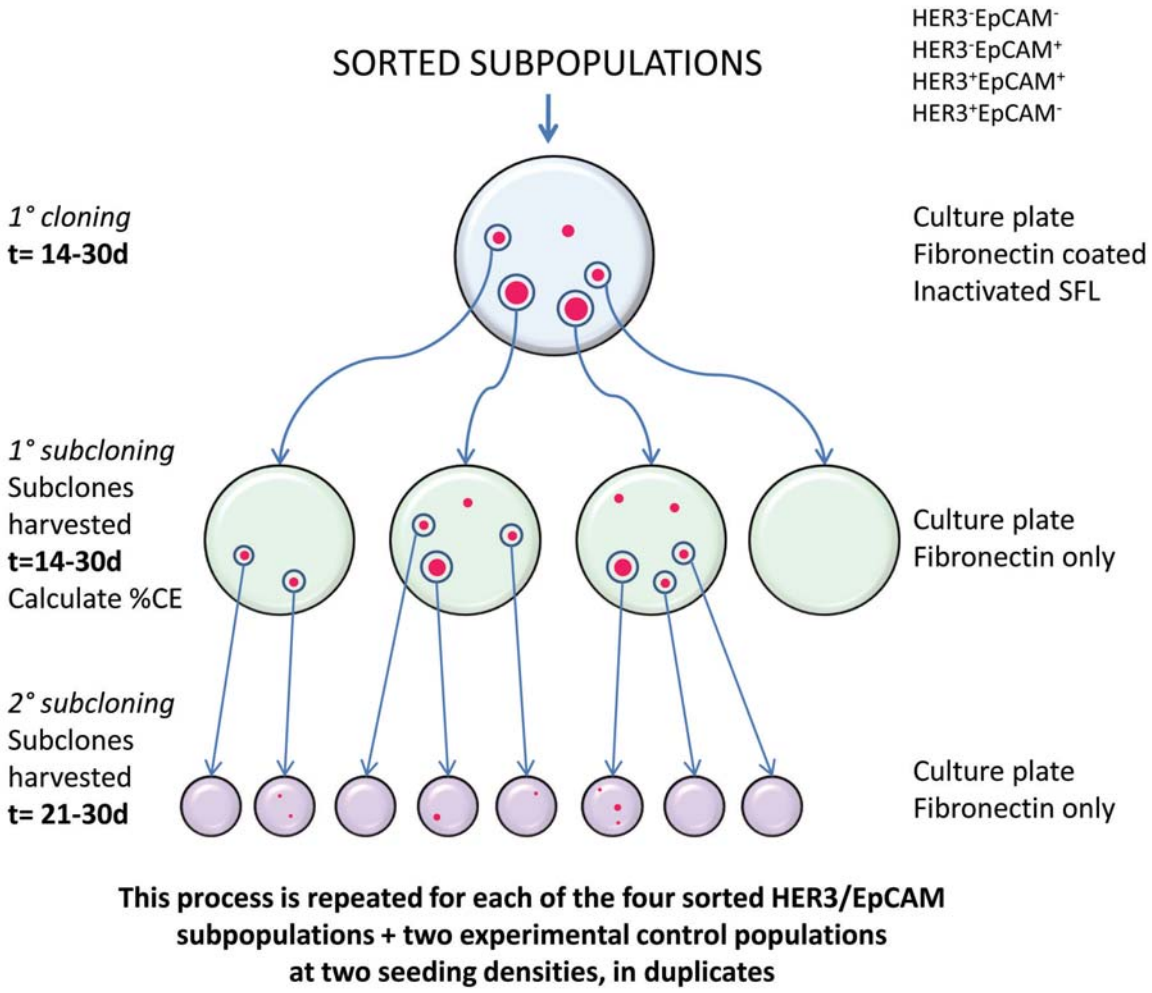


Figure 3.4: Protocol to assess clonogenicity (primary (1 °) cloning) and self-renewal (primary (1 °) and secondary (2 °) subcloning) *in vitro*. t, time in days and SFL, a stromal feeder layer was used.

of sorted cell fractions were varied in cell numbers (ie. 186–300,000 cells) thus amounts of extracted RNA (ie. 1–313ng) also varied. Where $\leq$500ng RNA was obtained, the maximum amount (11μl) was reverse transcribed with Superscript III and random primers (both Invitrogen). Absence of residual genomic DNA in the RNA preparations was verified by PCR on controls without reverse transcription (ie. No Template Control, NTC) and water only. cDNA reaction product was amplified with primers using GoTaq Green Master Mix (Promega, Madison, WI, USA) in a GeneAmp PCR system 2700 (Applied Biosystems, Forster City, CA, USA). Primer sequences used are shown in Table 3.5. Primer concentrations used were 10pmol. After amplification, reaction products were analysed by 1.5% agarose gel electrophoresis and stained with GelRed nucleic acid gel stain (1:2,000; Biotium, Hayward, CA, USA). Gels were imaged using Gel Doc XR+ system (Biorad, Hercules, CA, USA) and analysed using Image Lab software (2.0; Biorad). The sequence of each product was extracted using QIAquick gel extraction kits (QIAGEN, Hilden, Germany) according to manufacturer instruction and confirmed using automated sequencing at The Gandel Charitable Trust (Monash Health Translation Precinct, Clayton, VIC; previously done by S.Hubbard).

3.2.9 Statistical analysis

Data was analysed using GraphPad PRISM software (Version 5, San Diego, CA, USA). Gaussian distribution was examined using Kolmogorov–Smirnov test. Kruskal–Wallis tests were done for multiple colony-forming efficiency comparisons as sample numbers were small (or data was not normally distributed) followed by Dunn's multiple comparison test if data was significant. Mann Whitney *U* tests were used for comparison between two groups. Data are presented as mean $\pm$ SEM, unless stated otherwise. $P < 0.05$ was considered statistically significant.

Gene	Direction	Primer sequence	Tm	Cycles
NANOG	FWD	AAGACAAGGTCCCGGTCAAG	60	35
	RVS	CCTAGTGGTCTGCTGTATTAC		
SOX2	FWD	CCCCCCTGTGGTTACCTCTT	60	35
	RVS	TTCTCCCCCCTCCAGTTCG		
OCT4A	FWD	GAAGGTATTCAGCCAAAC	55	35
	RVS	CTTAATCCAAAAACCCTGG		
BMI	FWD	ATGTGTGTGCTTTGTGGAG	58	35
	RVS	AGTGGTCTGGTCTTGTGAAC		
CYTOKERATIN 8	FWD	ACCCAGGAGAAGGAGCAGCT	55	35
	RVS	CCGCCTAAGGTTGTTGATGT		
GAPDH	FWD	AGCCACATCGCTCAGACAC	64	40
	RVS	GAGGCATTGCTGATGATCTTG		

Table 3.5: Primer sequences for PCR amplification of self-renewal and pluripotent genes. FWD, forward; RVS, reverse; Tm, melting temperature ($^{\circ}$C) and Cycles, number of PCR cycles.

3.3 Results

To assess HER3 as a potential prospective marker of epithelial progenitors, fresh human endometrial epithelial cells were sorted into four subpopulations based upon the relative expression of HER3 and EpCAM. Colony-forming assays and reverse-transcription polymerase chain reaction (RT-PCR) were performed as screening tests to determine if any subpopulation was enriched for clonogenic and self-renewing cells.

3.3.1 HER3 is immunolocalised to glandular epithelia only

The location of putative human endometrial epithelial progenitor cells is unknown. To shed light on this, immunohistochemistry was carried out on full thickness human endometrial tissue in cycling and non-cycling endometrium (Fig 3.6A–C). HER3 showed greater expression in the basalis compared to functionalis (Fig 3.6D–H). Expression was immunolocalised to the luminal epithelium (Fig 3.6B) and basolateral surface of the glandular epithelium (Fig

3.6F–H) and was present at all stages of the menstrual cycle. During the proliferative stage of the menstrual cycle, some basal glands had weak and heterogeneous HER3 staining (Fig 3.6D), whilst others were negative (data not shown). Interestingly, the intensity of HER3 immunostaining in basal glandular epithelium was similiar between proliferative and inactive endometrium (Fig 3.6F, H). Expression of HER3 appeared greatest during secretory phase (Fig 3.6G). Matched isotype controls were negative (Fig 3.6G–I). EpCAM was used as a positive marker of endometrial epithelium (data not shown).

3.3.2 HER3 marker expression on freshly isolated human endometrial epithelial cells

To determine the utility of HER3 as a single marker to prospectively isolate epithelial progenitors from human endometrium, freshly isolated epithelial cells were analysed by flow cytometry using a two-colour protocol (Section 3.2.3, Fig 3.3). Epithelial cells were sorted into positive or negative subpopulations on the basis of HER3 expression (Fig 3.7A). Interestingly, the populations of $HER3^+$ ($31.7 \pm 8.7\%$, n=9) and $HER3^-$ ($42.3 \pm 8.1\%$, n=9) endometrial cells (Fig 3.7B) were similiar. To determine which subpopulation contained epithelial progenitor cells, cloning studies were used as a screening test. $HER3^+$ and $HER3^-$ subpopulations were seeded at cloning densities, however, there was no significant difference in $HER3^+$ (0.02 ± 0.016, n=9) and $HER3^-$ (0.009 ± 0.006, n=7, p=0.72) CFU activity (Fig 4.3C). Unforuntately, the majority of samples from both subpopulations failed to generate colonies, resulting in a large variation of CFU activity.

3.3.3 HER3/EpCAM marker expression on freshly isolated human endometrial epithelial cells

EpCAM, an epithelial marker has been used in combination with other surface markers for the identification of mammary and lung epithelial progenitor cells in human and mice

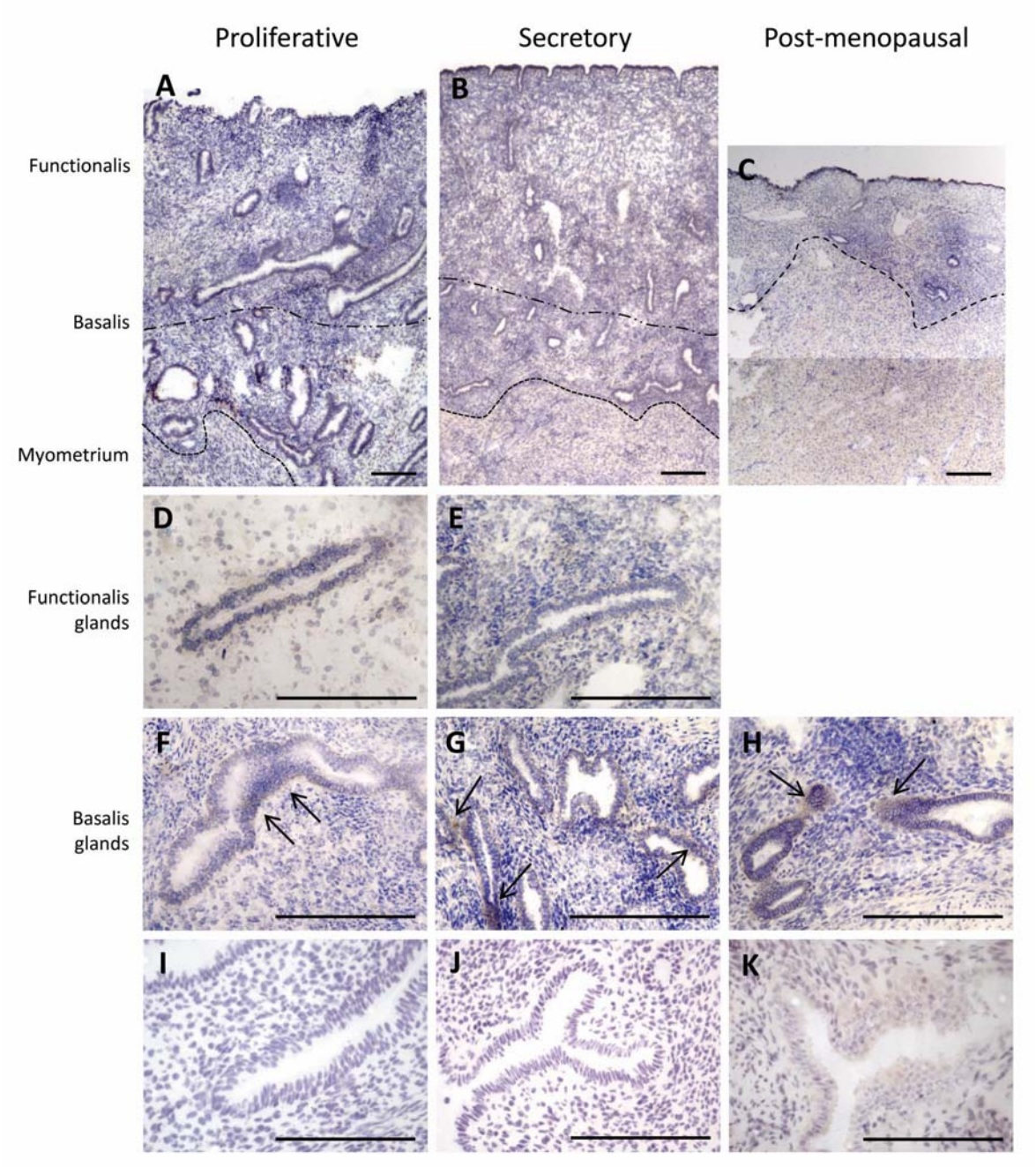


Figure 3.6: Endometrial glandular epithelia is positive for HER3 during (A, D, F) proliferative, (B, E, G) secretory stages of the menstrual cycle and in the (C, H) post-menopausal endometrium. Arrows point to heterogeneous staining. (G–I) Representative sections of isotype matched controls. Scale bar: 100μm.

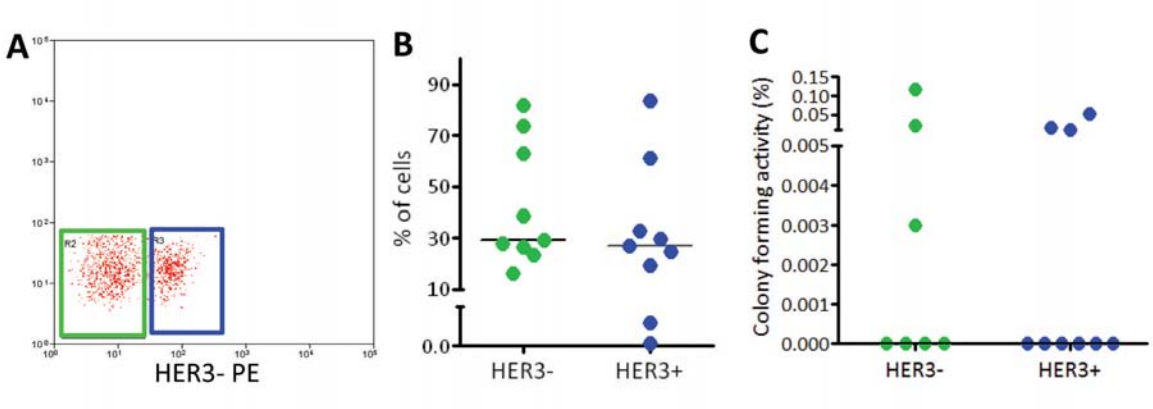


Figure 3.7: HER3 as a single candidate marker to prospectively enrich for endometrial epithelial progenitor cells. (A) HER3$^+$ (outlined in dark blue) and HER3$^-$ (outlined in green) populations were sorted by flow cytometry. (B) Percentage of cells expressing HER3$^+$ and HER3$^-$ by flow cytometry. Solid bar represents the median. (C) Comparing the cloning efficiency of HER3$^+$ and HER3$^-$ cells. Data is shown as a scatterplot, with each dot representing an individual sample.

(Stingl et al., 2001; Lim et al., 2009; McQualter et al., 2010). It was for this reason that the utility of HER3 and EpCAM was examined as co-markers for the prospective isolation of human endometrial epithelial progenitor cells. Freshly isolated epithelial cells were analysed by flow cytometry using a four-colour protocol (Section 3.2.3, Fig 3.3). Flow cytometric analysis revealed small populations of HER3$^+$EpCAM$^+$ (mean $\pm$ SEM, 7.2 $\pm$ 2.7%, n=6), HER3$^-$EpCAM$^+$ (13.74 $\pm$ 7.1%, n=5) with the smallest HER3$^+$EpCAM$^-$(1.7 $\pm$ 1.1%, n=5) albeit with a large range (Fig 3.8). The majority of endometrial cells did not express either marker (60.8 $\pm$ 11.7%, n=6).

3.3.4 Morphology and clonogenicity of HER3/EpCAM endometrial epithelial subpopulations

Sorted cell populations formed colonies with different morphologies. HER3$^-$EpCAM$^+$ cells formed basic polyhedral colonies with a mature epithelial phenotype (Fig 3.9C). HER3$^+$EpCAM$^+$ cells formed heterogeneous colonies of mature and immature epithelial phenotypes (Fig 3.9B).

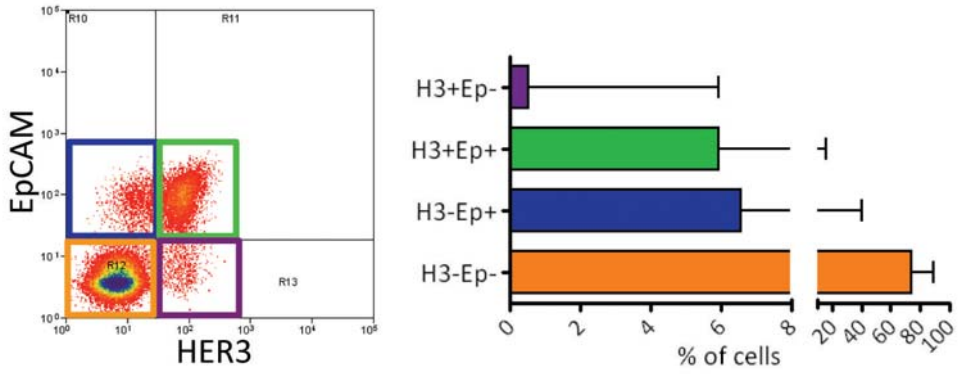


Figure 3.8: Percentages of HER3/EpCAM cells by flow cytometry. Bar graph representing median and range percentage of cells within each sorted subpopulation (n=6 for all, except $HER3^{-}EpCAM^{+}$ n=5). H3, HER3 and Ep, EpCAM.

Interestingly, $HER3^{+}EpCAM^{-}$ cells formed heterogeneous colonies of immature epithelial and round cell phenotypes (Fig 3.9D). These small round cells had a high nuclear:cytoplasm ratio.

Each endometrial epithelial subpopulation was assessed for clonogenicity, the ability of a single cell to give rise to a colony. Figure 3.9E shows the clonogenic activity of the four sorted epithelial subpopulations, which was highest for $HER3^{+}EpCAM^{-}$ ($0.85 \pm 0.45\%$, n=5), which interestingly was the smallest population (Fig 3.9B). This was a two- and 11-fold increase in comparison to $HER3^{+}EpCAM^{+}$ ($0.40 \pm 0.38\%$, n=5) and $HER3^{-}EpCAM^{+}$ ($0.07 \pm 0.03\%$, n=5) respectively (both p=0.095). Interestingly, the $HER3^{+}EpCAM^{+}$ subpopulation had three of the lowest CFU activity compared to all other subpopulations examined, severely reducing the mean clonogenicity. In the non-epithelial $HER3^{-}EpCAM^{-}$ subpopulation, clonogenicity was very high at $2.06 \pm 1.52\%$ (n=4). Although there was no difference between subpopulations for the enrichment of clonogenic epithelial CFU, a strong trend exists for HER3 and EpCAM as prospective markers (p=0.12).

Our laboratory group has previously demonstrated that large epithelial CFU albeit few in number, have higher proliferative potential and self-renewal ability than small CFU (Gargett et al., 2009). To examine this, the number of small and large CFU were counted and

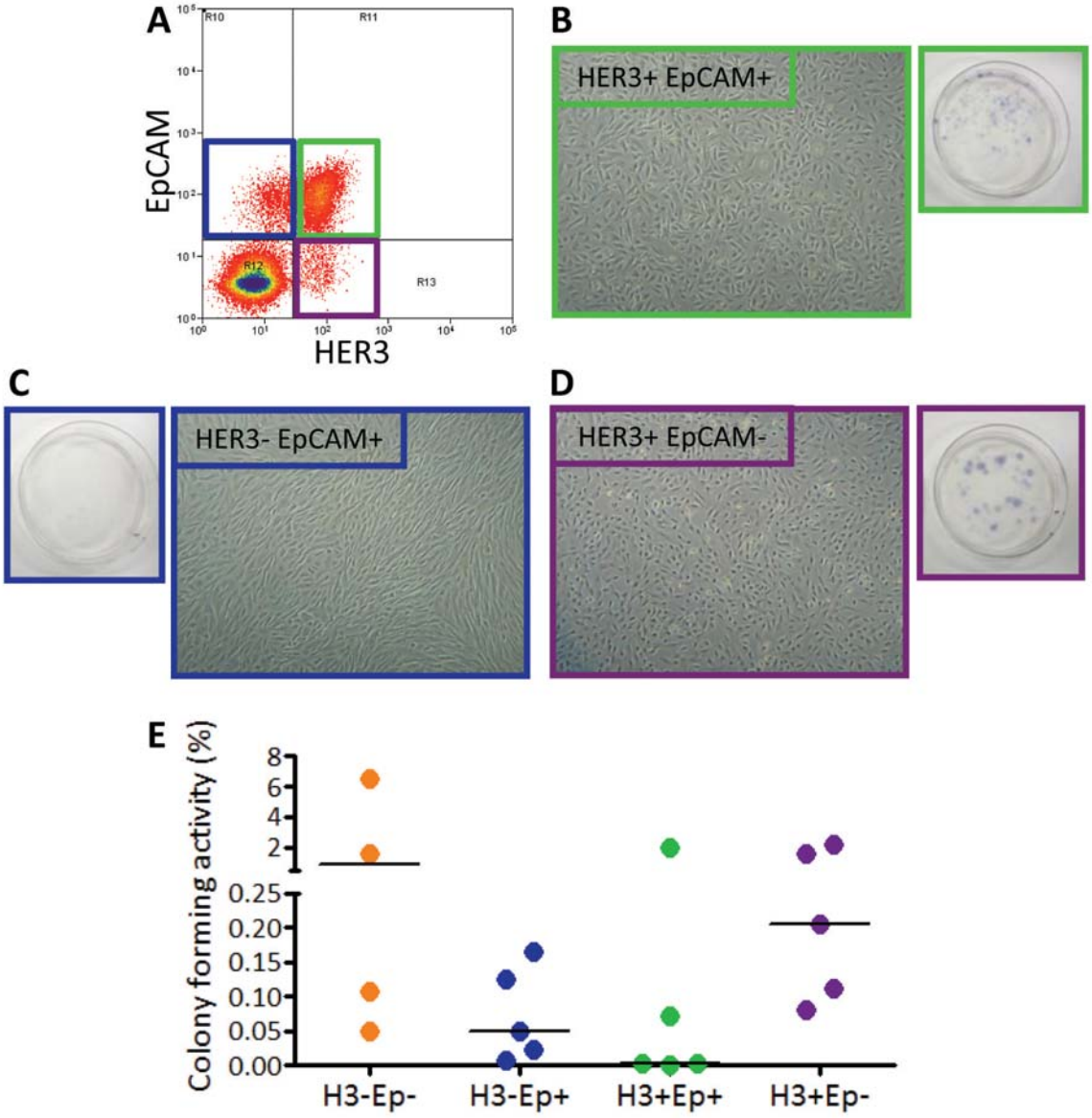


Figure 3.9: Human endometrial epithelial cells were sorted into subpopulations based upon relative expression of HER3 and EpCAM. Micrographs of representative epithelial colonies formed by (B) $HER3^{+}EpCAM^{+}$, (C) $HER3^{-}EpCAM^{+}$ and (D) $HER3^{+}EpCAM^{-}$ cells. (E) Epithelial subpopulations were assessed for clonogenicity. Solid bar represents median. Data is shown as a scatterplot, with each dot representing an individual sample. H3, HER3 and Ep, EpCAM.

compared for each subpopulation. Table 3.10 shows that there was a significant difference between the small and large colonies in terms of cloning efficiencies for HER3$^-$EpCAM$^-$ (p=0.03) and HER3$^-$EpCAM$^+$ (p=0.03) subpopulations. The large colonies formed by these subpopulations represented 0.4% and 13.8% of total colonies produced. Surprisingly, there was no significant difference between small and large clones in the HER3$^+$EpCAM$^+$ subpopulation (p=0.3), where the large colonies represented 8% of total epithelial colonies. Interestingly, HER3$^+$EpCAM$^-$ produced the most large epithelial clones, representing 18.7% of all colonies which was almost statistically significant (p=0.06).

	Small colonies	Large colonies	P value*	n
H3-Ep-	2.05 ± 1.52	0.008 ± 0.008	0.03	4
H3-Ep+	0.06 ± 0.03	0.0096 ± 0.0096	0.03	5
H3+Ep+	0.40 ± 0.38	0.035 ± 0.035	0.34	5
H3+Ep-	0.70 ± 0.35	0.161 ± 0.146	0.06	5
P value**	0.1	0.46		

Table 3.10: Mean cloning efficiences for small and large CFU for each subpopulation. Data are mean ± SEM. *, Mann Whitney and **, Kruskal-Wallis statistical tests were used.

3.3.5 Self-renewal of HER3/EpCAM endometrial epithelial CFU

Self-renewal of sorted HER3/EpCAM subpopulations was examined by serially cloning individual large CFU. The majority could be subcloned twice, indicating that CFU underwent self-renewing divisions to establish new clones (Table 3.11). HER3$^-$EpCAM$^-$, HER3$^-$EpCAM$^+$ subpopulations and sorted viable cells (7AAD$^-$/SYTOX Blue$^-$) had similar self-renewal activity, with the majority of clones from these populations undergoing one primary subcloning. The HER3$^+$EpCAM$^-$ population only subcloned twice which was surprising given this population produced the highest number of large colonies (Table 3.10) and was expected to be self-renewing. In contrast, all HER3$^+$EpCAM$^+$ subclones could be subcloned twice

and was the only subpopulation to be subcloned three times demonstrating that self-renewing CFU were enriched in this fraction. Sorted viable cells and unsorted cells were compared to identify any effects flow cytometry sorting had on cell survival. A proportion of unsorted fresh endometrial cell samples could be subcloned three times, however sorted viable cells could only be subcloned once. This indicated that flow cytometry sorting diminished epithelial cell survival and clonogenic and self-renewal activity.

Subpopulation	1°clone	2° clone	3° clone
H3-Ep-	4/6	1/3	0/2
H3-Ep+	5/6	1/3	0/2
H3+Ep+	5/6	3/3	2/2
H3+Ep-	5/6	2/3	0/2
Sorted viable	3/6	0/3	0/2
Unsorted	4/6	1/3	1/2

Table 3.11: The proportion of HER3/EpCAM subpopulations that produced clones. Number of patient samples that formed clones/total number of patient samples that were harvested as individual colonies/patient sample at each subcloning as described (Section 3.2.6). For each patient sample at each subcloning $\geq$3 clones were tested. 1 ° clone, primary cloning; 2 ° clone, primary subcloning and 3 ° clone, secondary subcloning.

3.3.6 Expression of pluripotency and self-renewal genes in HER3/EpCAM endometrial epithelial subpopulations

The expression of pluripotency, self-renewal (*NANOG*, *OCT4*, *SOX2* and *BMI1*) and differentiation associated (*CYTOKERATIN 8* (*CK8*)) genes were examined in HER3/EpCAM epithelial subpopulations by RT-PCR (Fig 3.12 and Fig 3.13). For this study, only qualitiative RT-PCR data was reported because the amount of RNA extracted from HER3/EpCAM subpopulations was minute and did not allow for fully reliable quantitiative RT-PCR analysis.

Results indicated expression of *CK8* and *SOX2* in all endometrial samples. *NANOG* and *OCT4* mRNAs were detected in both HER3^{-}EpCAM^{+} (n=2) and HER3^{+}EpCAM^{+} (n=1) samples but not in all HER3^{+}EpCAM^{-} samples (n=2). *BMI1* mRNA was expresesd in all samples with the exception of HER3^{+}EpCAM^{-}. However, the absence of *NANOG*, *OCT4* and *BMI1* in HER3^{+}EpCAM^{-} could be due to the small amounts of RNA extracted (ie. 1 and 3.16ng, n=2) as demonstrated by weak or lack of *GAPDH* expression, thus resulting in a false negative. Overall, there was no enrichment of pluripotent or self-renewal pathway genes in any flow cytometry sorted epithelial subpopulations using this semi-quantiative analysis and small sample size.

3.4 Discussion

For the first time, this study found that HER3 together with EpCAM enriches for endometrial epithelial CFU and enables the prospective isolation of endometrial epithelial progenitors. The HER3^{+}EpCAM^{-} subpopulation produced the greatest number of CFU and the greatest proportion of large colonies, however the HER3^{+}EpCAM^{+} subpopulation showed the greatest self-renewal ability. Pluripotent and self-renewal genes were expressed in the majority of HER3/EpCAM subpopulation samples. Although it was not possible to definitively discern which subpopulation was enriched for epithelial progenitor cells, this study is the first to prospectively isolate human epithelial clonogenic cells from the endometrium and provides novel data suggesting that HER3 may be a useful marker that partially purifies this population.

HER3 is a receptor in the epidermal growth factor signaling pathway (Casalini et al., 2004). Previously studies have concentrated on understanding its role in endometrial cancer rather than investigating the normal endometrium (Srinivasan et al., 1999; Ejskjaer et al., 2007). This study identified HER3 expression in the luminal epithelium and the basolateral surface of the glandular epithelium in normal tissue. There was stronger staining observed during the secretory compared to proliferative stage of the menstrual cycle, in agreement with published

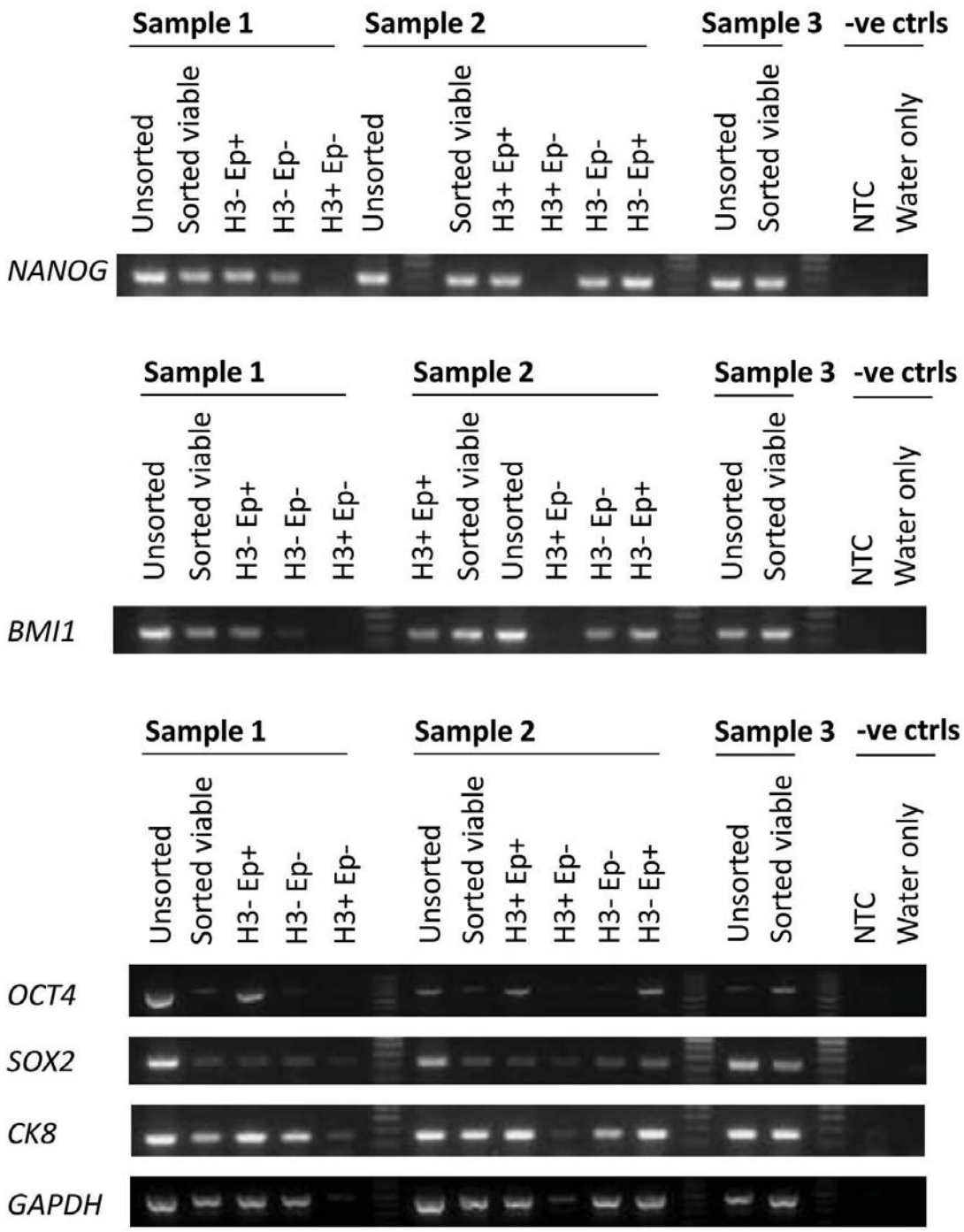


Figure 3.12: Expression of pluripotency, self-renewal and differentiation genes in endometrial epithelial HER3/EpCAM subpopulations by RT-PCR. All populations passed through the FACS sorter except for the unsorted cells. Sorted viable cells were 7AAD$^-$ or Sytox Blue$^-$. Negative controls (-ve ctrls) are No Template Control (NTC) and water only. H3, HER3 and Ep, EpCAM.

	Unsorted	Sorted viable	H3-Ep-	H3-Ep+	H3+Ep+	H3+Ep-*
NANOG	3/3	3/3	2/2	2/2	1/1	1/2
BMI1	3/3	3/3	2/2	2/2	1/1	0/2
OCT4	3/3	3/3	1/2	2/2	1/1	1/2
SOX2	3/3	3/3	2/2	2/2	1/1	2/2
CK8	3/3	3/3	2/2	2/2	1/1	2/2
GAPDH	3/3	3/3	2/2	2/2	1/1	1/2

Table 3.13: Qualitative RT-PCR analysis of pluripotent, self-renewal and differentiation-associated genes in endometrial epithelial HER3/EpCAM sorted subpopulations. Data reported as the proportion of samples showing gene expression/total number of samples examined. H3, HER3 and Ep, EpCAM. *, inadequate RNA extracted from this small subpopulation may explain lack of expression of some genes.

findings (Ejskjaer et al., 2005). Others however have reported expression of HER3 only during secretory stage and not during proliferative (Prigent et al., 1992), perhaps due to differences in the specificity of antibody clones used. In Chapter 2, two clones of HER3, 1D1D12 and 1B4C3 were compared, however the latter did not immunoreact as well with endometrial cells by both immunohistochemistry and flow cytometry. Previously, it was demonstrated that three out of eight HER3 peptides generated immunoreactive antibodies which failed to correlate with immunoblot results (Prigent et al., 1992), indicating the importance in selecting an appropriate HER3 epitope and antibody clone.

Very little is known about endometrial epithelial progenitors, including their origin and location. It has been hypothesised that human endometrial epithelial progenitor cells reside in the basalis (Gargett, 2007) in order to provide a source of cells that regenerate glands of the functionalis every menstrual cycle. However, several published reports have suggested that the regeneration of the endometrium is unlikely to come from the glandular epithelium (Baggish et al., 1967), instead arising from underlying stroma rather than as outgrowths from glandular epithelial cells (Garry et al., 2009). During menstruation, rapid early surface regeneration is known to occur (Garry et al., 2009), however no mitotic cell divisions took place in epithelial

glands of the basalis (Garry et al., 2010). Thus, it was concluded that glandular structures must arise by differentiation of neighbouring stromal cells which must comprise the source of stem/progenitors in the endometrium (Garry et al., 2010). Garry et al. (2009, 2010) used both hysterectomy and curettage samples, however did not report the proportions collected for study. The curettage technique generally removes a biopsy sample of the functionalis (Skinner et al., 1999; Salamonsen et al., 2001). This offers a possible explanation for the lack of mitotic cell divisions observed, as the deep basalis, the hypothesised location of the epithelial stem/progenitor cell niche may not have been collected and therefore could not be investigated (Masuda et al., 2010).

The current source of cells repairing the human epithelial lining following menstrual shedding is currently unknown. However, a study using a validated mouse model of endometrial breakdown and repair reported that glandular epithelial cells retain their bromodeoxyuridine-label for longer periods and selectively proliferate after repair, a very interesting finding suggesting that progenitor cells proliferate well after the regenerative response (Kaitu'u-Lino et al., 2010). In full thickness endometrium, stronger heterogeneous HER3 staining of glandular epithelium was observed in the basalis compared to functionalis in agreement with a previous study (Prigent et al., 1992). Furthermore, there were several very strong HER3 expressing gland profiles in the deep basalis near the endometrial-myometrial interface. Future studies to identify whether the hormone responsive epithelial label-retaining cells of the mouse model of endometrial breakdown and repair express HER3 would further validate this marker as specific for endometrial epithelial progenitors. In addition, this study found similar levels of expression in inactive and proliferative stage endometria indicating that HER3 positive cells do persist throughout a lifetime and are located near the hypothesised endometrial stem/progenitor cell niche. Clonogenic epithelial cells have also been demonstrated in inactive endometrium (Schwab et al., 2005). It would be interesting to investigate whether clonogenic epithelial cells from inactive endometrium express HER3, and whether they express pluripotent and self-renewal genes. Although this would be technically challenging as

very few cells can be isolated from an atrophic endometrium.

The novel combination of HER3 and EpCAM substantially enriched for endometrial epithelial progenitors, resulting in a colony-forming capacity two- and four-fold greater in the HER3^{+}EpCAM^{-} and HER3^{+}EpCAM^{+} subpopulations than the previously reported endometrial epithelial cloning efficiency (Chan et al., 2004). These subpopulations formed colonies that varied in morphology, forming small and large phenotypes. The majority of large epithelial CFU were found in the HER3^{+}EpCAM^{-} and HER3^{+}EpCAM^{+} subpopulations, an important observation as large colonies have been reported to contain a greater number of self-renewing CFU (Gargett et al., 2009).

The colony-forming assay was used as a screening test. This was followed by subcloning, a more stringent time-consuming assay to assess self-renewal activity, a key adult stem cell property (Weissman et al., 2001; Marley et al., 2003; Gargett et al., 2009). Results indicated that HER3^{+}EpCAM^{+} may be enriched for endometrial epithelial progenitors, as primary colonies were able to subclone three times. HER3^{-}EpCAM^{-} and HER3^{-}EpCAM^{+} subpopulations, although clonogenic could not be subcloned a second time indicating that progenitors were unlikely to be enriched in these subpopulations and were therefore of no interest to this study.

The expression of several self-renewal and pluripotency genes suggests the possible presence of stem cells, as these genes are essential for maintaining stem cell function (Cai et al., 2004; Takahashi and Yamanaka, 2006). However, embryonic stem cell gene expression is not conserved in all adult stem cell populations (Forte et al., 2009). Nevertheless, this study demonstrated HER3/EpCAM *CK8*$^{+}$ cells also expressed several pluripotent and self-renewal genes, *BMI1*, *NANOG*, *OCT4* and *SOX2* albeit with a limited sample size. *SOX2* was expressed in all endometrial samples in agreement with previous findings (Wong et al., 2010; Götte et al., 2011), although in contrast to Forte et al. (2009), who reported the absence of *SOX2*. This discrepancy could be possibly due to PCR technique differences. Others have also reported the expression of *OCT4* in endometrium (Matthai et al., 2006; Cervello et al., 2010; Bentz et al.,

2010). However this is a highly contentious marker of stemness as the expression of *OCT4* has also been detected in differentiated adult blood cells (Zangrossi et al., 2007). Therefore, the expression of *OCT4* is not sufficient to define pluripotency as the many pseudogenes of *OCT4* can produce false positive results (Zangrossi et al., 2007; Monk et al., 2008; Wang and Dai, 2010), highlighting the importance of correct primer design. To prevent false positives, this study designed primers that avoided the amplification of multiple psuedogenes (Monk et al., 2008). Similar to previous reports (Forte et al., 2009), expression of *BMI1* was detected in all endometrial samples with exception of the $HER3^{+}EpCAM^{-}$ subpopulation which is likely due to the insufficient RNA extracted from this minute subpopulation. These markers have also been found in endometrial carcinoma and endometriosis (Hubbard et al., 2009; Götte et al., 2011), suggesting their origin may have been from one of these HER3/EpCAM subpopulations.

The limitations of this study are related to difficulties associated in working with human samples. The availability of samples and adequate size to provide sufficient numbers of endometrial cells ($\geq 1x10^{6}$) for flow cytometry sorting were not in abundance at various times during this project. This was due to the introduction and increased used of Mirena, a progesterone releasing intrauterine device which halved the number of available hysterectomies for study. Adding to this, the rarity of progenitor cells in somatic tissues makes a difficult task even more challenging to obtain substantial cell counts from flow cytometry sorting for further experimentation such as cell culture and RT-PCR. It is not unusual for only several hundreds of $HER3^{+}EpCAM^{-}$ cells to be collected by flow cytometry sorting. As CFU are rare, statistically there may not have been sufficiently enough cells sorted to realistically expect one or more self-renewing CFU. A possible solution is to combine patient samples together. However, matching age and stage of menstrual cycle would not be possible as samples are collected prospectively from surgically resected tissues. Also, the use of purified single cells removed from the support of their *in vivo* neighbouring cells is a limitation as the epithelial cells may behave differently (Coulombel, 2004). During initial assessment of HER3

as a single prospective marker, sorted HER3$^+$ cells were not cultured on inactivated stromal feeder layers and this could explain the absence of colonies *in vitro* in the majority of samples. Consequently, further *in vitro* clonal studies were carried out with inactivated stromal feeder layers which resulted in HER3/EpCAM cell colony formation. These observations indicate the importance of stromal-epithelial interactions (Cunha and Lung, 1979; Kurita et al., 2001) in supporting the *in vitro* culture of epithelial cells.

In this study, several epithelial HER3$^+$EpCAM$^{+/-}$ subpopulations had overall higher self-renewal ability than sorted viable cells, demonstrating that viability was not a determining factor of self-renewal and that the *in vitro* results obtained were real. Comparisons between sorted viable and unsorted cells revealed greater self-renewal in the latter indicating that flow cytometry sorting is a harsh process and does affect cell recovery and survival. This reinforces the significance of the self-renewal ability of the HER3$^+$EpCAM$^+$ subpopulation which were able to undergo three subclonings despite being flow cytometry sorted. These observations are in agreement with other published findings (Rivkin et al., 1986; Emre et al., 2010) where the addition of p160-Rho-associated coiled kinase (ROCK) inhibitor, Y-27632 improved post-sorting recovery in embryonic stem cells (Emre et al., 2010). The ROCK family are effectors involved in cell morphology, motility, proliferation and apoptosis whose signaling pathways are currently being elucidated (Riento and Ridley, 2003; Rikitake and Liao, 2005; Liao et al., 2007). Future *in vitro* studies could investigate the use of Y-27632 to improve the culture of flow cytometry sorted endometrial epithelial cells. Alternatively, anti-IgG magnetic beads could be used to isolate marker-expressing subpopulations rather than passing the fragile cells through a flow cytometer.

The HER3$^+$EpCAM$^+$ and HER3$^+$EpCAM$^-$ subpopulations hold much promise as enriched populations of endometrial epithelial progenitors, although any potentially significant differences were possibly masked due to the small sample size. It is unfortunate that a minute quantity of RNA was extracted from the HER3$^+$EpCAM$^-$ subpopulation, possibly accounting for the absence of *OCT4*, *NANOG* and *BMI1*, thus, making it difficult to determine

which subpopulation had the greatest enrichment. Nevertheless, the high clonogenicity and self-renewal activity combined with the expression of pluripotent and self-renewal genes strongly suggests the presence of progenitors in the HER3^{+}EpCAM$^{+/-}$ subpopulations. It is acknowledged that although very promising, more samples are required to identify which HER3/EpCAM subpopulation is enriched for endometrial epithelial progenitor cells.

This is the first study to prospectively isolate a small subpopulation of epithelial cells from the human endometrium demonstrating clonogenic activity, self-renewal and pluripotency gene expression. These clonogenic and self-renewing cells are likely to be located in the basal layer and are present in the inactive endometrium, thus persisting for a lifetime. The ability to prospectively isolate these epithelial progenitors provides the opportunity for further characterisation and to find more specific markers to further increase purity of these rare cells. It also supports future investigations into the possible role of endometrial epithelial progenitors in gynaecological disorders such as endometriosis and endometrial cancer.

3.5 Supplementary Information

3.5.1 Endometrial tissue collected and isolated but insufficient counts for FACS sorting

A hindrance to this study's progress was the small size of a number of samples. Supplementary Table 3.14 lists samples that once isolated into single cell suspensions, did not provide sufficient cell counts for further investigation.

Sample	Age	Menstrual Stage	Pathology
23-09	48	ES	Menorrhagia
58-09	50	ES	Leiomyomata
60-09	31	Disordered P	Menorrhagia
64-09	47	Menstrual day 1	Dysmenorrhoea
65-09	48	S	Menorrhagia
74-09	50	Menstrual day 2	Menorrhagia
83-09	48	P	Menorrhagia
87-09	48	P	Enlarged uterus
130-09	45	MS/LS	Leiomyomata

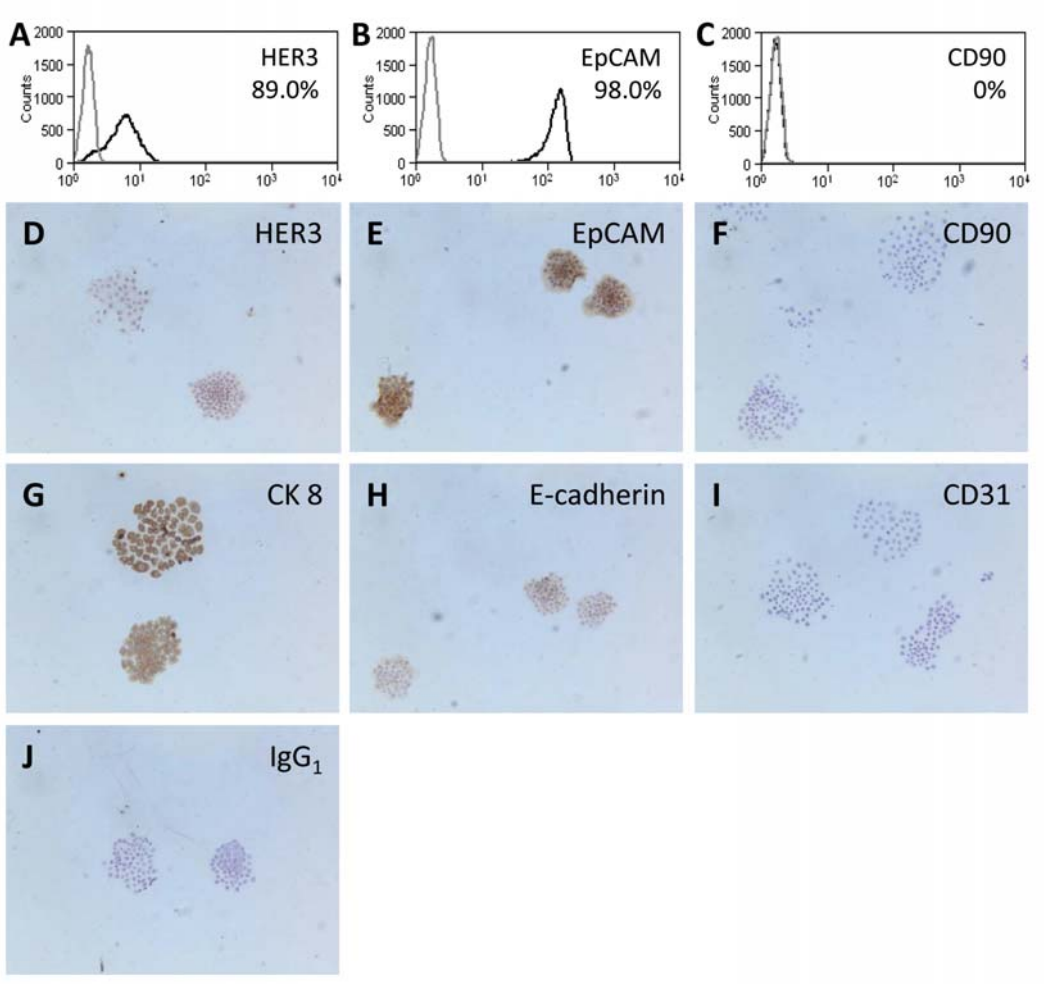
Supplementary Table 3.14: Samples from which cells were isolated but not further experimented upon. Menstrual stages: proliferative (P), secretory (S), early secretory (ES), mid-secretory (MS), late secretory (LS).

3.5.2 ECC-1, endometrial epithelial cancer cell line as a positive control

ECC-1, an endometrial carcinoma cell line maintains a luminal epithelial phenotype and expresses both estrogen, both progesterone and androgen receptors (Mo et al., 2006) and was used as a suitable positive control for flow cytometry and immunocytochemistry analysis.

ECC-1 cells were immunolabelled for flow cyometry as previously described 3.2.3. Briefly, ECC-1 cells were incubated with antibodies against HER3 (250μl/ml dilution; H-J.Bühring), EpCAM (5μg/ml; goat anti-human IgG; R&D Systems) and APC-conjugated anti-CD90 (5μg/ml; BD Pharmingen). For immunocytochemistry, ECC-1 were cultured on coverslips in 4-well plates (both NUNC A/S, Roskilde, Denmark) in bicarbonate-buffered DMEM/F12 medium containing 10% FCS, 10ng/ml epidermal growth factor (human recombinant; BD Biosciences), 2mM glutamine (Invitrogen) and Primocin (100μg/ml; Invivogen) at 37 °C in 5% CO_2 for 5 days. Cells were fixed with acetone (Merck) for 2 min at 4 °C, incubated with antibodies against CK8 (used supernatant neat, mouse anti-human IgG_1; low molecular weight, clone 35βH11, Dako), E-cadherin (250μl/ml dilution, optimised in Chapter 3; H-J.Bühring), EpCAM (11μg/ml; mouse anti-human IgG_1; clone Ber-EP4; Dako), CD90 (1μg/ml; BD Pharmingen) and CD31 (1:75; Dako). Matched isotype control mouse IgG_1 (Dako Cytomation) were used at equvialent concentrations for negative controls. Cells were imaged as previously described (Section 2.2.4).

A large proportion of ECC-1 cells were positive for HER3 (Suppl Fig 3.15A,D) and EpCAM (Suppl Fig 3.15B,E). Immunocytochemistry detected CK 8 (Suppl Fig 3.15G) and E-cadherin (Suppl Fig 3.15H) but not CD90 (Suppl Fig 3.15C,F) and CD31 (Suppl Fig 3.15I) on ECC-1 cells.



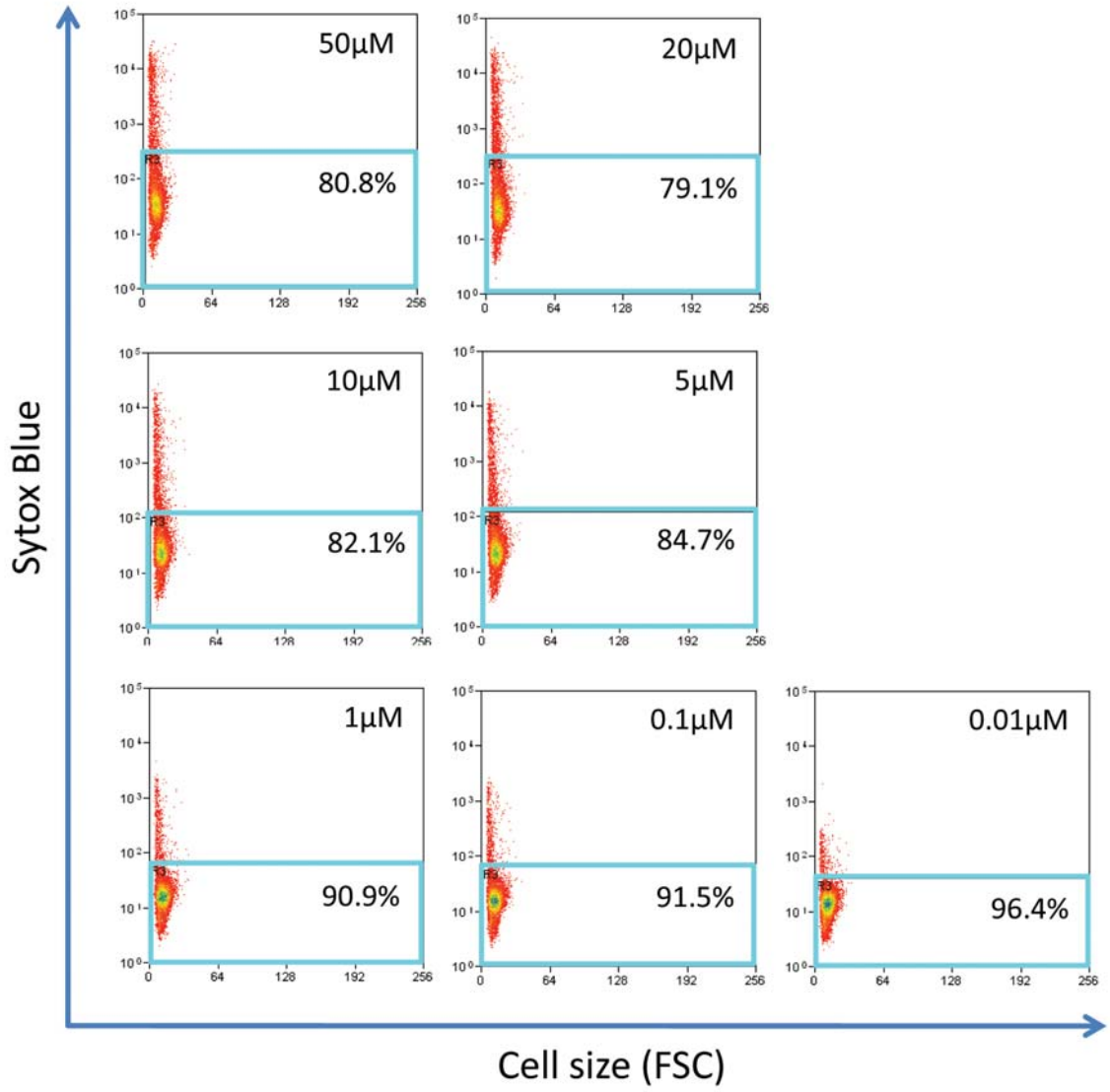
Supplementary Figure 3.15: ECC-1 cell line expresses HER3. (A) Single parameter flow cytometry histogram for HER3, (B) EpCAM, and (C) CD90 with percentage of positive cells (n=1). Black line: antibody. Grey line: isotype control. Immunostaining for (D) HER3, (E) EpCAM, (F) CD90, (G) CK 8, (H) E-cadherin, (I) CD31 and (J) IgG_1.

3.5.3 Optimisation of SYTOX Blue

SYTOX Blue dead cell stain is a high-affinity nucleic acid stain that penetrates cells with compromised plasma membranes such as non viable cells (Molecular Probes, Invitrogen). Conventional 7AAD (Sigma-Aldrich) dead cell stain requires an incubation time of 30 min and could possibly be toxic to endometrial epithelial cells. In contrast, SYTOX Blue only requires a 5 min incubation time, thereby exposing cells to less potential toxicity and shortened protocols. Additionally, SYTOX Blue did not require flow cytometry colour compensation with the multi-colour protocol established in this study.

Optimisation of SYTOX Blue was done using endometrial cells isolated from a curette sample #158-10 by methods previously described (Section 3.2.2). Freshly isolated endometrial cell suspensions (1x10^5 cells) were aliquoted into seven tubes, containing Flow Buffer (100μl, 2% fetal calf serum (FCS; Invitrogen)/PBS) and incubated with seven different concentrations (0.01, 0.1, 1, 5, 10, 20, 50μM) of SYTOX Blue (Molecular Probes Invitrogen). Following a 5 min incubation with SYTOX Blue, samples were immediately analysed using a MoFlo flow cytometer (DakoCytomation, Fort Collins, CO, USA) and Cyclops SUMMIT software (Version 5.2; DakoCytomation). Cells were selected for analysis by electronically gating the forward versus side scatterplot and for single cells. A minimum of 10,000 events were collected. Viability was determined by electronic gating of viable cells using the parameters of cell size (forward scatter) versus SYTOX Blue expression (Suppl Fig 3.16). Viable cells are reported in percentages (n=1).

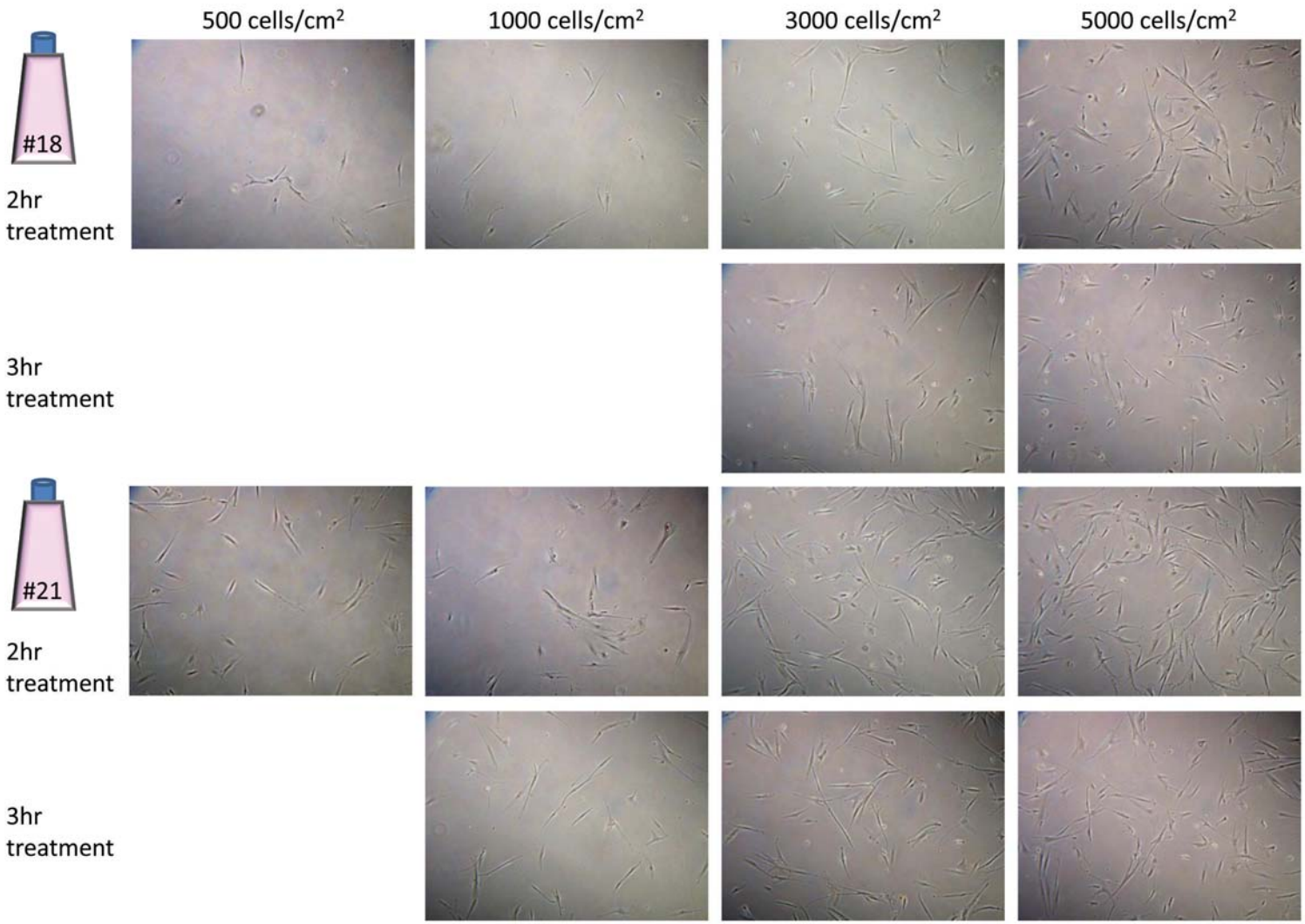
SYTOX Blue at 1μM concentration proved to be efficient in seperating between viable and non-viable cells and falls within the manufacturers recommendation.



Supplementary Figure 3.16: Optimisation of SYTOX Blue on freshly isolated endometrial cells. Viable cells are outlined in blue.

3.5.4 Optimisation of stromal feeder layers

Intitally a human endometrial stromal cell line (HESCs) (Krikun et al., 2004) at 2 different passages (referred to as #18-HESCs and #21-HESCs) was tested as a potential stromal feeder layer for co-culture of FACS sorter epithelial cell subpopulations. Mitomycin-C (10μg/ml; Sigma-Aldrich, St. Louis, MO, USA) was used to inactivate the feeder layer with treatment time and seeding densities to be optimised. Two and three hour treatment durations and 4 seeding densities (500, 1000, 3000, 5000 cells/cm^2) in duplicates were tested (Suppl Fig 3.17). To determine which treatment duration was effective, 3 day cultured inactivated HESCs were trypinsied and counted to ensure that no proliferation had taken place. Mitomycin-C inactivation for two hours proved to be efficient in preventing HESCs proliferation and seeding at 3000 cells/cm^2 ensured an equal distribution of feeder cells in petri dishes.



Supplementary Figure 3.17: Optimisation of stromal feeder layers. Two different passages of HESCs were Mitomycin-C treated for two or three hours and seeded at four densities. A two hour treatment and seeding at 3000 cells/cm^2 was selected for co-culturing with sorted epithelial cells.

CHAPTER 4

Endometrial stem/progenitor cells and endometriosis

4.1 Introduction

Endometriosis is a common gynaecological disorder that is characterised by growth of endometrial tissue in an ectopic location outside the uterus. The pathogenesis of endometriosis remains unknown however common belief is that retrograde reflux of menstruation carries viable fragments of endometrium that deposit in the peritoneal cavity and establish ectopic lesions (Sampson, 1927). Although a majority (90%) of women experience retrograde reflux (Halme et al., 1984), only a minority (6-10%) develop the disorder (Giudice and Kao, 2004). A possible explanation is that endometrial stem/progenitor cells are abnormally shed during menstruation and reflux into the peritoneal cavity where they establish to form lesions in women who develop endometriosis (Leyendecker et al., 2002; Gargett and Chan, 2006; Sasson and Taylor, 2008; Gargett and Masuda, 2010; Maruyama et al., 2010).

There have been no reports on the direct role of endometrial/stem progenitor cells in endometriosis. However, unfractionated menstrual blood has been examined as a convenient source of mesenchymal stem cells for regenerative therapies (Cui et al., 2007; Meng et al.,

2007; Hida et al., 2008; Patel et al., 2008). Another potential accomplice in the development of endometriosis is peritoneal fluid (PF), primarily an exudation product of the ovaries (Koninckx et al., 1980) and is in contact with all organs contained within the peritoneal cavity. Previous studies have reported endometrial gland-like structures and cells in the PF of women with and without endometriosis (Bartosik et al., 1986; Kruitwagen et al., 1991; van der Linden et al., 1995). Endometrial cells obtained from PF were analysed by cytologic methods or bulk cultured (Willemsen et al., 1985; Bartosik et al., 1986; Kruitwagen et al., 1991; van der Linden et al., 1995) however no cloning studies to assess stem cell activity have been done. Others have flushed the uterine cavity to recapitulate retrograde reflux and demonstrated an increased presence of endometrial epithelial cells in the PF (Willemsen et al., 1985; Bartosik et al., 1986). Following tubal flushing, an increase of endometrial tissue was found in the PF from women with and without endometriosis (Bartosik et al., 1986) suggesting that whilst flushing recapitulates retrograde menstruation, it may not necessarily represent what occurs naturally and may not provide useful information as to the cause or initiation of endometriosis.

This study aims to investigate shedding endometrium and peritoneal fluid collected from menstruating women with endometriosis and women without endometriosis as controls, for the presence of clonogenic endometrial cells by *in vitro* assay and expression of putative and known stem/progenitor cell markers by flow cytometry. The hypothesis of this work is that endometriotic lesions are initiated by stem/progenitor cells that have been erroneously released into the peritoneal cavity during menstruation.

4.2 Materials & Methods

4.2.1 Patient Criteria

Women were aged 34.1 $\pm$ 0.9 yrs (range 23–42 yrs, ages of two patients were unknown). For menstruating samples, women with endometriosis (n=5) or those undergoing diagnostic

	Age (yrs)	Day of menstrual cycle	Stage of disease
Control patients			
CP1	31	2	—
CP2	31	2	—
CP3	38	2	—
CP4	42	2	—
CP5	28	3	—
CP6	31	3	—
CP7	33	3	—
CP8	37	Un	—
CP9	Un	3	—
CP10	28	6	—
CP11	36	7	—
CP12	35	21	—
CP13	37	24	—
CP14	28	28	—
CP15	35	28	—
Endometriosis patients			
EP1	29	2	1
EP2	39	2	2
EP3	39	2	4
EP4	34	3	1/2
EP5	37	3	2
EP6	38	14	1
EP7	23	20	4
EP8	40	28	1
EP9	39	28	4
EP10	35	Un	1
EP11	Un	Un	1
EP12	30	Un	4

Table 4.1: Patient characteristics. Un, unknown.

laparoscopy or tubal ligation without endometriosis (controls; n=8) were recruited on days 1–3 of their menstrual cycle (during menstruation) (Table 4.1).

For non-menstruating controls, cycling women were recruited with (n=4) and without (n=6) endometriosis. The presence or absence of endometriosis was determined by visual inspection of the peritoneal cavity by the gynaecologist (Dr. Gareth Weston) collecting the samples. Photographic evidence was taken during surgery and disease severity was classified

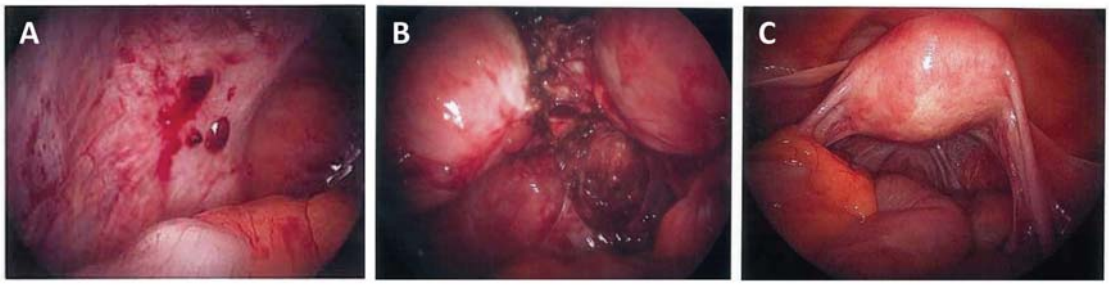


Figure 4.2: Representative laparoscopy photos of women with (A) mild endometriosis (Stage 1–2), (B) severe endometriosis (Stage 3–4) or (C) without endometriosis (controls). The presence and absence of endometriosis was determined by the gynaecologist. Endometriosis was classified using the Revised American Ferility Society Classification of Endometriosis (ASRM, 1997).

using the Revised American Fertility Society Classification of Endometriosis (ASRM, 1997) by the gynaecologist (Fig 4.2).

Recruited patients kept a menstrual diary tracking their menstrual cycles for 2–3 months to establish regularity. Once menstrual patterns had been established, patients were scheduled for a laparoscopic surgery on day 2 of their period for collection of samples by the gynaecologist. Some patients were serendipitously recruited if they were menstruating at the time of laparoscopy. Classification of patients occurred on the day of surgery, only women who were within 1–3 days of menstruation were considered menstruating and women on days 6–28 of the menstrual cycle were considered non-menstruating. Ethics approval was obtained from the Monash Medical Centre Human Research and Ethics Committee and informed written consent was obtained from each patient.

4.2.2 Patient samples collected

Uterine menstrual effluent (uterine menstrual blood, UMB) was collected by gentle aspiration of the cavity using sterile soft tubing attached to a syringe. Similarily, vaginal menstrual effluent (vaginal menstrual blood, VMB) was aspirated in the same manner from the vaginal cavity. VMB represents shedding endometrium that had collected in the vagina and was

contaminated with vaginal squamous epithelia (Fig. 4.3). Undiluted peritoneal fluid (PF) was collected from all patients by syringe aspiration through a needle port prior (under the guide of a laparascope camera) to any flushing. On several occasions when there was very little PF, the peritoneal cavity was flushed via the laparoscope and flushing collected. Endometriotic lesions were excised from two patients, one non-menstruating severe endometriosis (two lesions) and one menstruating mild endometriosis (three lesions). Peripheral blood (PB) was also taken from the patient prior to anaesthesia, where possible. All samples collected, with the exception of the endometriotic lesions were transferred into blood collection tubes (Lithium-Heparin; BD Vacutainer, New Jersey, USA) . Sample volumes were recorded when possible and were diluted with a measured volume of Collection Medium (DMEM/F12 with HEPES; 1% Antibiotics, both Invitrogen, Carlsbaad, CA, USA; 2% fetal calf serum, Gibco/Invitrogen). Endometriotic lesions were collected separately into Collection Medium. Samples were processed within 2–18 hr of collection.

4.2.3 Preparation of single cell suspensions from peritoneal fluid and peripheral blood

Erythrocytes and dead cells were removed from PF and PB samples using Ficoll-Paque density gradient centrifugation medium (GE Healthcare, Uppsala, Sweden) (Chan et al., 2004). Prior to Ficoll-Paque centrifugation, PF and 4 ml of PB was diluted with 10 ml of Bench Medium (DMEM/F12 with HEPES; 5% newborn calf serum; 1% Antibiotics; Invitrogen, Carlsbaad, CA, USA) and PF was diluted with Bench Medium to make a final volume of 14 ml. Samples were centrifuged at 1500 rpm for 30 min with no brake at 4 °C. Cells for experimentation were removed from the Ficoll-Paque-medium interface, washed and resuspended in Bench Medium for counting (Fig 4.3) (Chan et al., 2004).

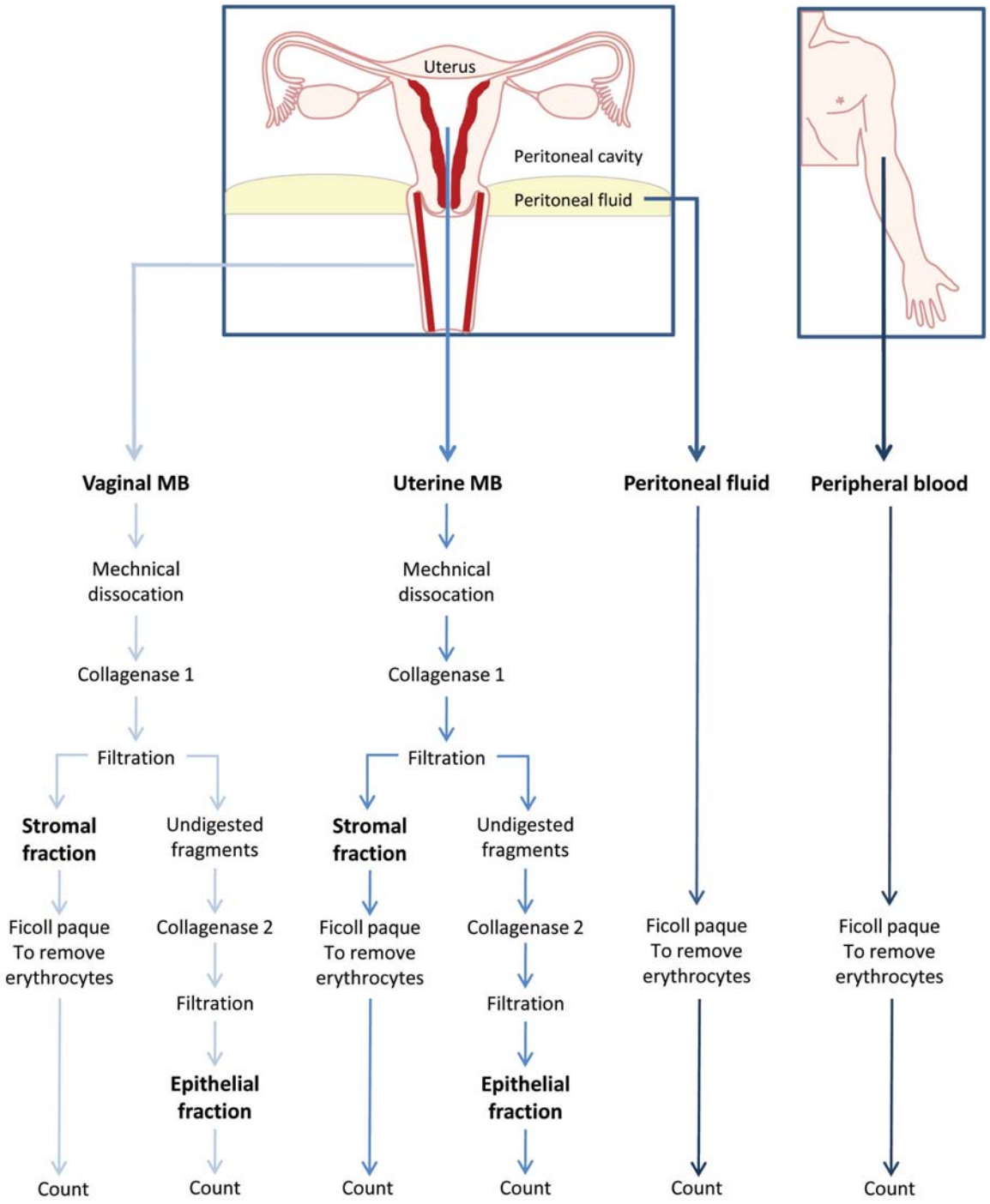


Figure 4.3: Samples collected from the endometrium, peritoneal cavity and venous system and the protocol to prepare them for assessment by *in vitro* clonal studies and flow cytometry analysis. MB, menstrual blood (shedding endometrium).

4.2.4 Preparation of single cell suspensions from vaginal, uterine menstrual blood and endometriotic lesions

Menstrual effluent contained fragments of shedding endometrium which was firstly dissociated into a single cell suspension as described in Chapter 2 (Section 2.2.2) with minor modifications. Briefly, tissue was mechanically dissociated using scissors followed by enzymatic digestion in Collagenease type 1 (5mg/ml; Worthington Biochemical Corporation, New Jersey, USA)/PBS and DNase1 (0.5mg/ml; Worthington Biochemical Corporation)/PBS for 1.5 hr and filtered to crudely separate stromal cells (filtrate) and epithelial gland fragments (filtrand). Glandular tissue (filtrand) underwent a second enyzmatic digestion with Collagenase type 2 (4mg/ml; Worthington Biochemical Corp)/PBS, DNase1 (1mg/ml; Worthington Biochemical Corporation)/PBS and Hyaluronidase (5mg/ml; Sigma-Aldrich, St. Louis, MO, USA)/PBS for 30–40 min (Fig 4.3) followed by filtration to produce a single-cell suspension. Erythrocytes and dead cells were removed by Ficoll-Paque as above (Section 4.2.3.1). Cells from endometriotic lesions were prepared using the same protocol for menstrual effluent however cells isolated by the first and second enzymatic digestions were combined and not separated.

4.2.5 Optimising collection and preparation of samples for experimentation

Given this was a new study, the protocol for both surgical collection of samples as well as isolation of cells from these samples was continuously refined for the first few samples.

4.2.5.1 Surgical collection of samples

Patients were usually recruited during their initial specialist consultuation at contraception and infertility clinics at Monash Medical Hospital, Monash Surgical Private Hospital and

Western Day Surgery. Logistically, collection of samples from women at days 1–3 of their menstrual cycle was difficult as it required co-ordinating the women's day of cycle (based upon menstrual diary) with the gynaecologist's operating list. To make this possible our method was refined to follow women using a menstrual diary during the 3–6 month surgery waiting list to allow a more precise prediction of when menses was due for suitable study participants.

During surgery where possible, PB was collected prior to the patient being anaesthetised to avoid the dilution of the PB by the intravenous administration of saline which is routine in surgical procedures. In the early stages of the study, the surgeon trialed washing the peritoneal cavity with saline and this was collected as PF washing. However, after some consideration it was decided that this would not provide indication of which cells were naturally present in PF, since uterine-tubal flushing causes artificial flushing of endometrial cells into the peritoneal cavity (Willemsen et al., 1985; Bartosik et al., 1986). Therefore, the protocol was refined for collection of undiluted PF. This required the presence of a dedicated surgeon who was prepared to change the order of normal operating procedures to allow for collection of samples which was time consuming and could result in delays in surgery lists for subsequent patients.

4.2.5.2 Laboratory isolation of samples

Endometrial cell isolation protocols had to be modified (Section 2.2.2) for menstrual blood and ectopic endometrial lesions, as shedding endometrium contained a lot of mucous and blood clots, and cauterised tissue surround the lesions had to be manually removed.

4.2.6 *In vitro* colony-forming assay to assess clonogenicity

Freshly isolated cells were assessed for clonogenicity as previously described in Chapter 3 (Section 3.2.5) and Gargett et al. (2009) with a minor modification that freshly isolated cells were seeded at cloning densities of 50, 100 and 200 cells/cm^2.

4.2.7 Immunolabelling cells for flow analysis

Freshly isolated cells were immunolablled for flow cytometric analysis as previously described in Chapter 2 (Section 2.2.3) with minor modifications. Antibodies used were raised against HER3 (supernatant, 250μl/ml; clone D1D12; mouse anti-human IgG$_1$; H-J.Bühring), EpCAM (11.8μg/ml; clone Ber-EP4; mouse anti-human IgG$_1$; Dako Cytomation, Glostrup, Denmark) or IgG$_1$ isotype control (Dako Cytomation) which were subsequently incubated with PE-conjugated rat anti-mouse IgG$_1$ (1μg/ml; BD Pharmingen, San Diego, CA, USA). Cells incubated with W5C5 (supernatant, 200μl/ml; mouse anti-human IgG$_1$; H-J.Bühring) were subsequently incubated with APC-conjugated rat anti-mouse IgG$_1$ (1μg/ml; Caltag, Invitrogen, Carlsbad, CA, USA) or APC-conjugated IgG$_1$ isotype control (1μg/ml; mouse anti-human; BD Pharmingen). Cells incubated with CD146 (supernatant, 500μl/ml; clone CC9; P.Simmons, University of Texas Health Science Center at Houston, Houston, TX, USA) were subsequently incubated with FITC-conjugated rat anti-mouse IgG$_{2A}$ (1μg/ml; Caltag, Invitrogen). Cells were also incubated with APC-conjugated CD90, CD31, CD45 (all 1μg/ml; BD Pharmingen) or FITC-conjugated Mesothelin (100μl/ml; monoclonal rat anti-human IgG$_{2A}$; R&D Systems, Minnneapolis, MN, USA).

4.2.8 Immunohistochemistry

Freshly isolated cells were seeded at 250–500 cells/cm^2 on coverslips (Thermanox, Nunc, Roskilde, Denmark) in 4-well plates (Nunc) in Culture Medium (bicarbonate-buffered DMEM/F12 containing 10% FCS, 10ng/ml Epidermal Growth Factor (human recombinant; BD Biosciences), 2mM glutamine (Invitrogen), Primocin (100μg/ml; Invivogen)) at 37 °C in 5% CO$_2$. Medium was changed every 2–3 days and monitored for 7 days or until 60–80% confluence. Cultured coverslips were immunostained as previously described in Chapter 2 (Section 2.2.4) with minor modifications. Cells were fixed by acetone for 2 min at 4 °C. Cultured coverslips were subsequently incubated with 0.3% hydrogen peroxide

(Orion Laboratories, Welshpool, Australia) and protein blocking agent (Thermo Electron Corp, Pittsburgh, U.S.A) for 10 min each at room temperature. Primary and isotype control antibodies were diluted in 0.1%BSA/PBS, and incubated for 1 hr at 37 °C. Antibodies used were α-smooth muscle actin (2.3μg/ml; clone IA4; mouse anti-human IgG $_{2A}$; Dako Cytomation), CA 125 (6μg/ml; clone OC 125; mouse anti-human IgG $_1$; Zymed/Invitrogen), low molecular weight Cytokeratin 8 supernatant (used neat; clone 35βH11; mouse anti-human IgG $_1$; Dako Cytomation), CD10 (4.5μg/ml, mouse anti-human IgG $_1$, Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) , CD31 (4.6μg/ml; mouse anti-human IgG $_1$; clone JC70A; Dako Cytomation), CD90 (4μg/ml, Thy-1; clone 5E10; BD Pharmingen), pan Cytokeratin (6.7μg/ml; mouse anti-human IgG $_1$; clone C-11, Sigma). Mouse isotype controls IgG $_1$ (Dako Cytomation) and IgG $_{2A}$ (Chemicon, Massachusetts, U.S.A) were used.

4.2.9 Statistical analysis

Data was analysed using GraphPad PRISM software (Version 5, San Diego, CA, USA). Gaussian distribution was examined using Kolmogorov–Smirnov test. Mann Whitney *U* tests were used for comparison between two groups. Data are presented as mean $\pm$ SEM, unless stated otherwise. P <0.05 was considered statistically significant.

4.3 Results

4.3.1 Clonogenicity of endometriotic lesions

To confirm that endometriotic lesions contained stem/progenitor cells, lesions were examined for CFU activity on a total of five lesions were collected from two patients with endometriosis. These lesions were individually processed into single cell suspensions and were assessed for clonogenicity *in vitro*. All five lesions demonstrated colony-forming ability (Fig. 4.4A) and formed heterogeneous colonies comprising epithelial (Fig. 4.4B) and mesenchymal cells (Fig.

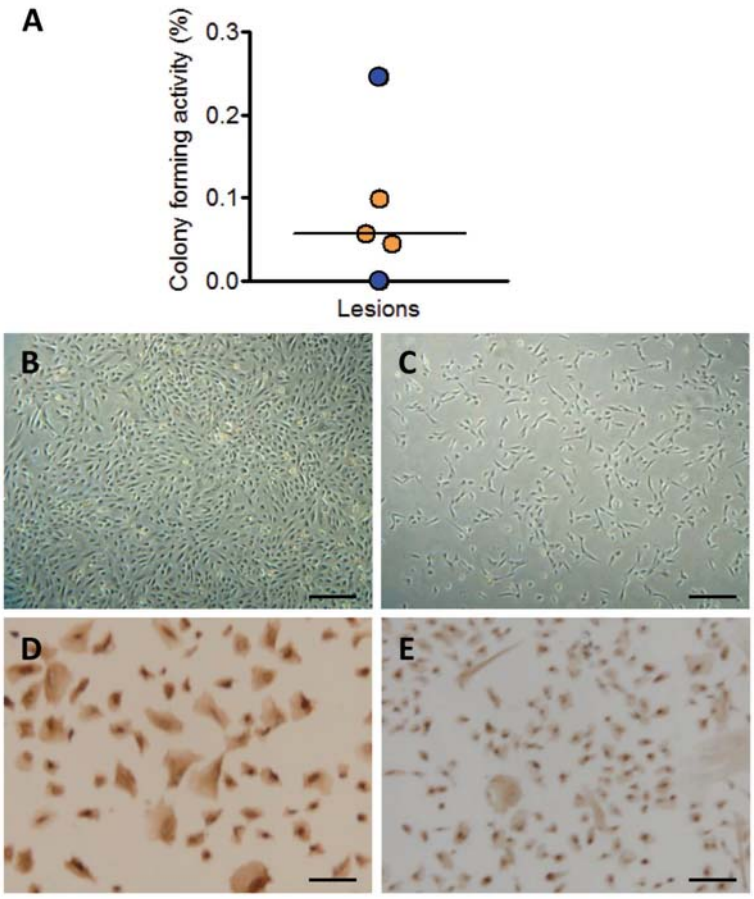


Figure 4.4: Cells isolated from endometriotic lesions were assessed for (A) clonogenicity. Data is shown as a scatterplot. Blue dots represent patient 1, orange dots represent patient 2 with each dot representing individual lesions. Solid bar represents median. Micrographs of representative (B) epithelial and (C) mesenchymal colonies formed *in vitro*. Immunostaining of cultured cells (D) Cytokeratin 8 and (E) CD90. Scale bar: 50μm.

4.4C) that were immunopositive for cytokeratin 8 (epithelial marker; Fig. 4.4D) and CD90 (mesenchymal/fibroblast marker; Fig. 4.4E) respectively.

4.3.2 Clonogenicity of menstrual blood collected from uterine and vaginal cavities, and peripheral blood

To establish that shedding endometrium contained endometrial stem/progenitor cells, manual syringe aspiration of the uterine cavity (uterine menstrual blood, UMB) and vaginal cavity

(vaginal menstrual blood, VMB) was collected during surgery of endometriosis and control women. Unfractionated UMB from women with endometriosis (n=3) and controls (n=1) were individually prepared into single cell suspensions and assessed for clonogenicity; however no apparent difference was observed for the small number of samples examined (Fig. 4.5A). Subsequently, the isolation method was refined and UMB was separated into two populations (epithelial and mesenchymal) by crude enrichment using filtration to assess clonogenicity of both cell types, given that endometrial mesenchymal colony-forming efficiency (CE) is over five times that of endometrial epithelial cells (Chan et al., 2004). However, there were no significant differences in the clonogenicity of epithelial (p=0.34, Fig. 4.5B) and mesenchymal (p=0.86, Fig. 4.5C) cells between menstruating endometriosis (n=4) and control (n=4) women. The number of samples collected from menstruating women and controls assessed for epithelial and mesenchymal clonogenicity is unequal because one UMB mesenchymal enriched fraction sample was lost to contamination. The epithelial fraction of this UMB sample has been represented as a green dot point (Fig. 4.5B).

Cells isolated from UMB formed homogeneous and heterogeneous colonies characteristic of epithelial (Fig. 4.6A) and mesenchymal cells (Fig. 4.6B). These cells were profiled by immunohistochemistry and were cytokeratin 8^+ (Fig. 4.6C), $CD90^+$ (Fig. 4.6D), weakly $CD10^+$ (Fig. 4.6E) and $CD31^-$ (Fig. 4.6F).

Unfractionated VMB was initially cultured with no apparent difference observed in endometriosis (n=2) and control (n=2) women (Fig. 4.7A). VMB was then crudely separated by filtration into epithelial enriched and mesenchymal enriched cell fractions and assessed for clonogenicity (Fig. 4.7B,C). In terms of clonal capacity, no apparent differences were found between endometriosis (n=4) and control (n=2) patients. It was unfortunate that two VMB mesenchymal enriched fractions were lost to contamination. The epithelial enriched fraction of these VMB samples has been represented as green dot points (Fig. 4.7B).

Given reports of bone marrow-derived stem cell contribution to endometriosis (Taylor, 2004; Du and Taylor, 2007), PB was collected from patients and assessed for colony-forming

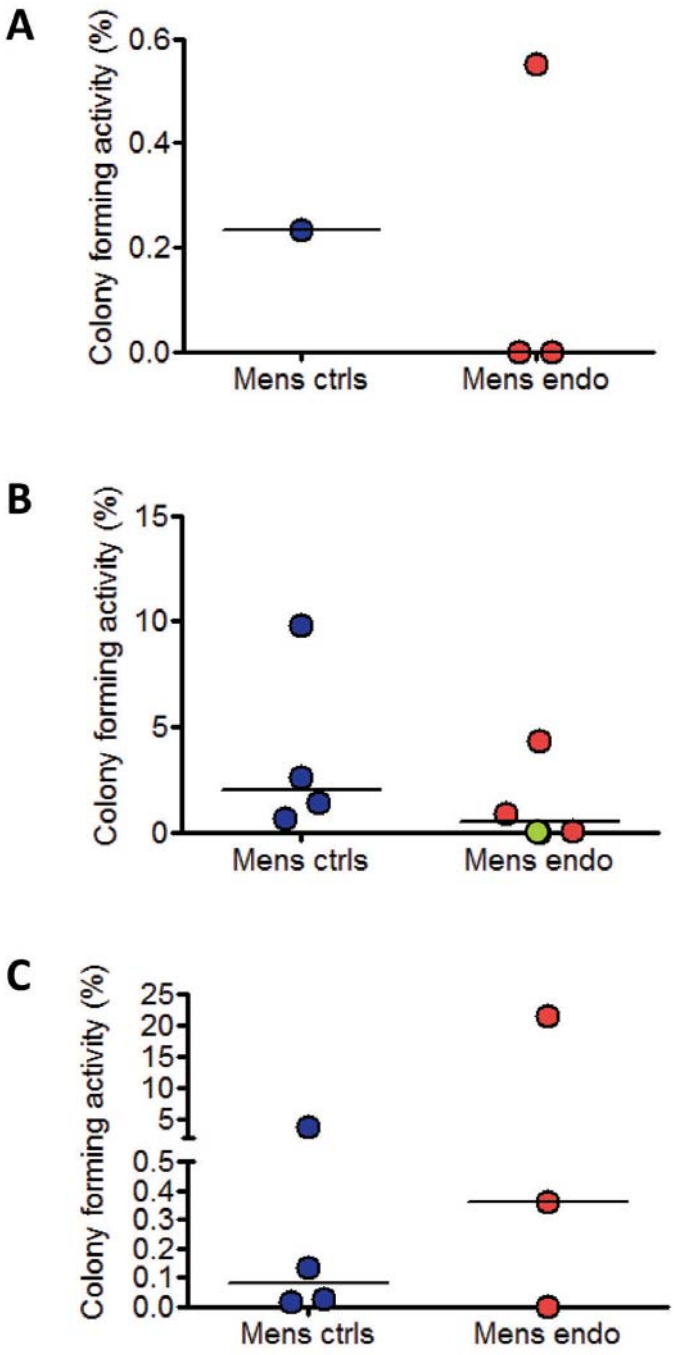


Figure 4.5: Shedding endometrium collected from the uterine cavity (UMB) was assessed for clonogenic endometrial cells. (A) Clonogenicity was initially assessed in unfractionated UMB. Thereafter UMB was separated into (B) epithelial enriched and (C) mesenchymal enriched fractions. Green dot point in panel B represents a sample where the UMB mesenchymal fraction was lost to contamination. Data is expressed as a scatterplot, with each dot representing an individual woman. Solid bar represents median. Mens endo, menstruating endometriosis patients and mens ctrls, menstruating control patients.

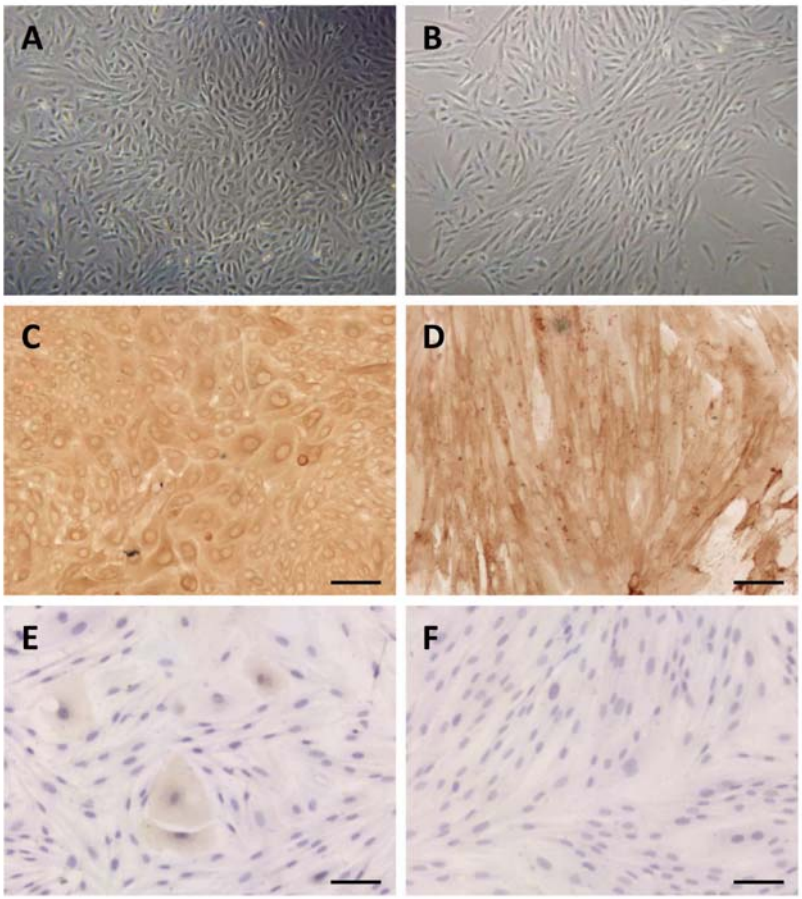


Figure 4.6: Cell cultures of shedding endometrium collected from the uterine cavity of endometriosis women and normal controls. Morphology of representative heterogeneous colonies of primarily (A) epithelial or (B) stromal cells formed *in vitro*. Immunostaining of cultured coverslips for (C) cytokeratin 8, (D) CD90, (E) CD10 and (F) CD31. Scale bar: 50μm.

activity since bone marrow-derived stem cells must be distributed in the circulation. However, PB samples collected from menstruating endometriosis (n=3) and control (n=4) women demonstrated no clonogenic ability (Fig. 4.7D).

4.3.3 Clonogenicity of peritoneal fluid

To investigate for a difference in PF volumes in menstruating women with and without endometriosis, undiluted PF was compared. There was a significant difference between

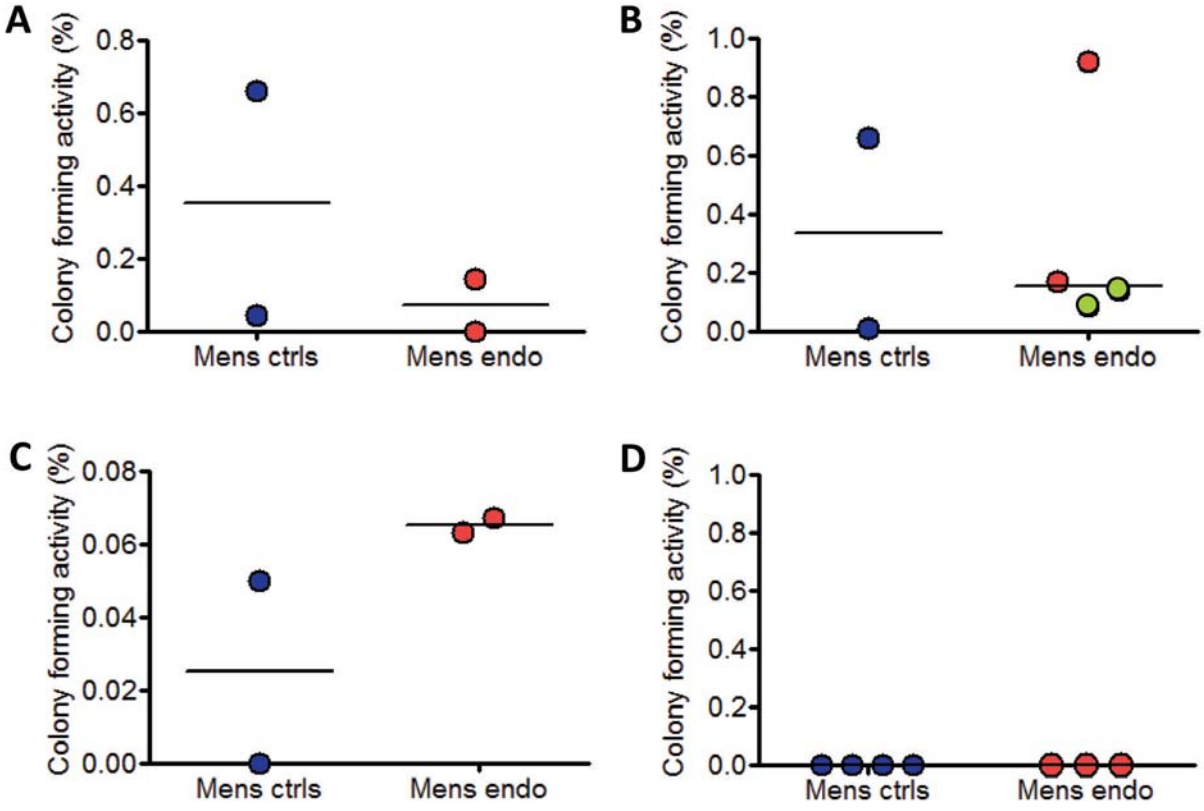


Figure 4.7: Shedding endometrium collected from the vaginal cavitiy (VMB) and peripheral blood were assessed for clonogenic endometrial cells. (A) Clonogenicity was initially assessed in unfractionated VMB. Thereafter VMB was separated into crude (B) epithelial enriched and (C) stromal enriched fractions. Green dot points in panel B represent samples where the VMB stromal fraction was lost to contamination. (D) PB. Data is expressed as a scatterplot, with each dot representing an individual woman. Solid bar represents median. Mens endo, menstruating endometriosis and mens ctrls, menstruating controls.

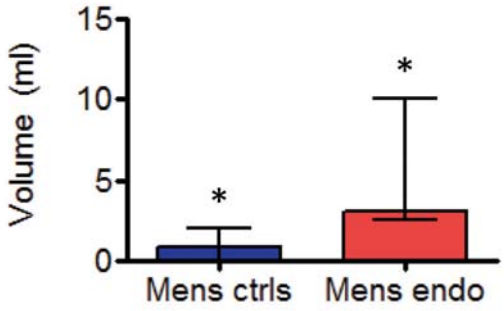


Figure 4.8: Volume of peritoneal fluid collected from menstruating women with and without endometriosis. Data is shown as a median with the range. Mens endo, menstruating endometriosis and mens ctrls, menstruating controls. *, Significance was tested using Mann Whitney (p=0.03).

the volumes of undiluted PF collected from women with endometriosis (n=4) compared to controls (n=4, p=0.03; Fig. 4.8).

Cells obtained from the PF of menstruating women with endometriosis (n=7) and controls (n=4), demonstrated clonogenic ability *in vitro* 0.07% and 0.05% respectively, however no significant difference was identified between the two groups (p=0.9; Fig. 4.9). All types of endometrial colonies were counted as one because epithelial colonies were rarely observed, with the exception of one patient with stage 2 endometriosis (Fig. 4.9 data point coloured in green, Fig. 4.10A), which interestingly demonstrated the highest clonogenic activity. Peritoneal mesothelial cell clones were also observed in the majority of PF samples and were more common than endometrial epithelial cell clones from women with and without endometriosis (data not shown).

There were obvious characteristic differences between adherent cells in culture. Cells isolated from PF were heterogeneous and morphologically characteristic of endometrial epithelial (Fig. 4.10A), endometrial mesenchymal (Fig. 4.10B) and peritoneal mesothelial cells with some mature or senescent cells (Fig. 4.10C,D). The same types of cells were found in endometriosis and control patient cultures.

Cultured cells from PF of endometriosis and control women were immunoreactive for

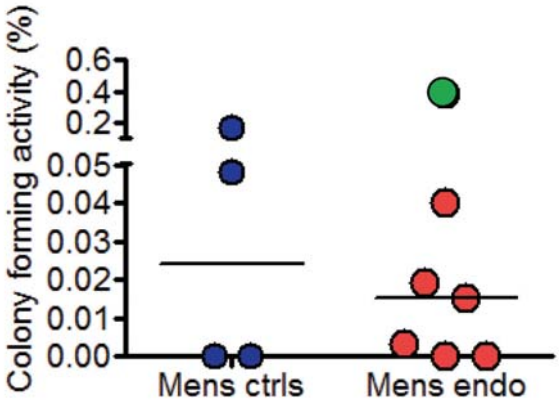


Figure 4.9: Peritoneal fluid from menstruating women was assessed for clonogenic endometrial cells. Data is shown as a scatterplot, with each dot representing an individual woman. All dot points represent mesenchymal cells with exception of the green dot point which represents epithelial and mesenchymal cells. Solid bar represents median. Mens endo, menstruating endometriosis and mens ctrls, menstruating controls.

Pan CK (Fig. 4.10E). Morphologically characteristic endometrial cells were mostly, but not always positive for CD10 (Fig. 4.10F) and CD90 (Fig. 4.10G), despite having similar morphologies. Morphologically characteristic endometrial cells were consistently negative for CA 125 (Fig 4.10H) and α-smooth muscle actin (Fig. 4.10J).

4.3.4 Expression of endometrial stem/progenitor cell markers

Samples collected from endometriosis and control women were characterized by flow cytometry for expression of putative and known endometrial stem/progenitor cell markers (Suppl Table 4.13). When comparing the UMB-epithelial fraction from women with endometriosis to controls, there was a 1.9- and 3.4-fold increase in HER3$^+$ and W5C5$^+$ cells (Fig. 4.11A, Suppl Fig. 4.12A) respectively. Similarily, when comparing the UMB-mesenchymal fraction from women with endometriosis to controls, there was a 1.8- and 6.4-fold increase in HER3$^+$ and W5C5$^+$ cells (Fig. 4.11B, Suppl Fig. 4.12B) respectively. When PF of menstruating endometriosis women was compared to controls, there was a 1.3- and 3.7-fold increase in expression of HER3 and W5C5 respectively, despite the small sample size (Fig. 4.11C, Suppl

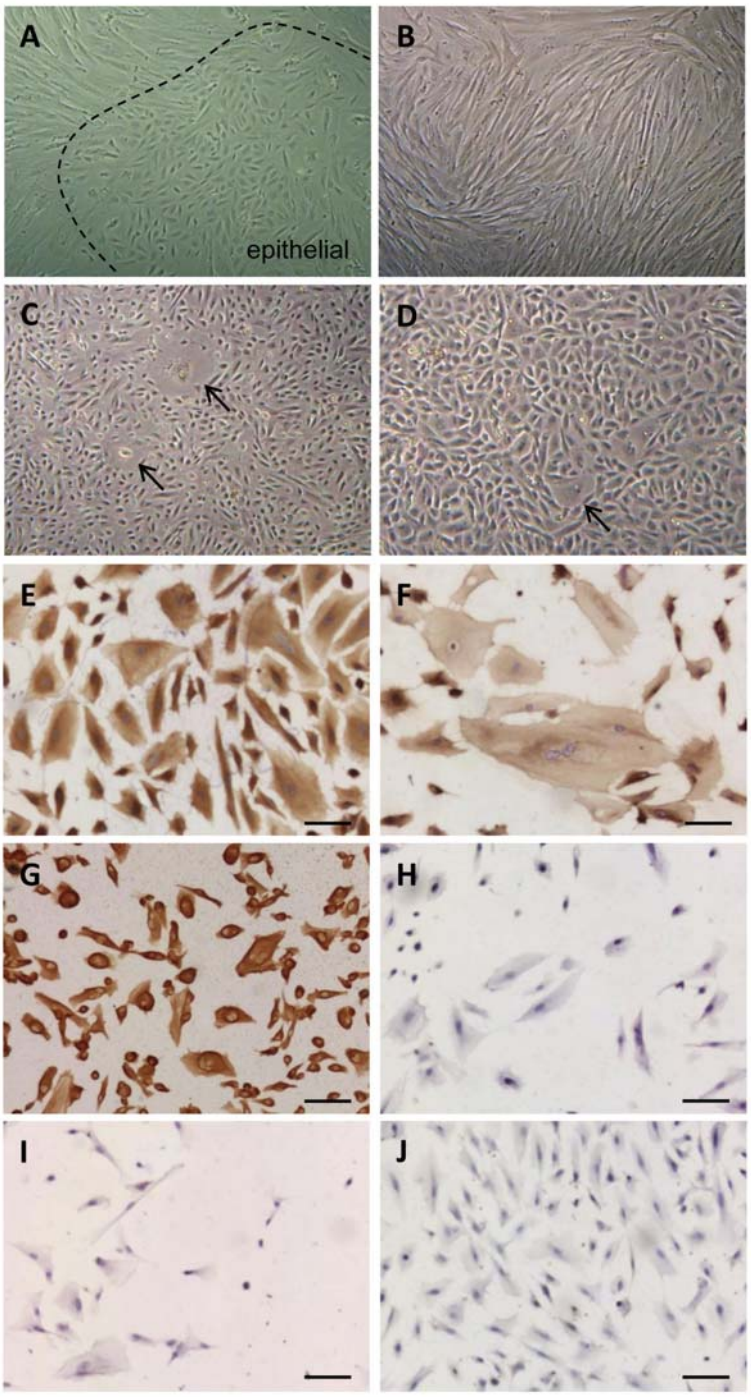


Figure 4.10: Morphology and immunostaining of cultured cells found in peritoneal fluid. (A) An epithelial colony overlaps with a mesenchymal colony (B) mesenchymal and (C, D) mesothelial cells were observed. Arrows indicate senescent cells. (E) Pan CK, (F) CD10, (G) CD90, (H) CA 125, (I) CD31 and (J) α-smooth muscle actin. Scale bar: 50μm.

Fig. 4.12C). Surprisingly, the expression of CD146, an endothelial marker that has been used as a marker of endometrial mesenchymal stem cells was not consistent (Schwab and Gargett, 2007; Schwab et al., 2008). When comparing data of CD146 from women with endometriosis and controls, a 1.4-fold increase was observed in the UMB-epithelial fraction (Fig. 4.11A, Suppl. Fig. 4.12A) with a 1.1- and 5- fold reduction in the UMB-stromal fraction and PF respectively (Fig. 4.11A,C, Suppl Fig. 4.12A).

4.4 Discussion

The main findings of this study are the increased expression of putative and known endometrial stem/progenitor cell makers in women with endometriosis compared to control women during menstruation. Suprisingly, no significant differences were found in the clonogenicity of endometrial stem/progenitor cells from shedding endometrium or PF of women with and without endometriosis however, clonogenic endometrial cells from endometriotic lesions were observed *in vitro*. This suggests that the hypothesis that shedding endometrium contains viable endometrial stem/progenitor cells that are effluxed into the peritoneal cavity during menstruation may not necessarily be confined to women with endometriosis as it occurs in most women.

Emerging research has identified putative cell markers that have enabled prospective isolation of endometrial stem/progenitor cells (Schwab and Gargett, 2007) (Chapter 3). Screening of freshly processed samples collected from menstruating endometriosis and control women with these markers HER3, W5C5 and CD146 by flow cytometry (Fig. 4.11), showed that cells in PF and shedding endometrium from endometriosis women had increased expression of HER3 and W5C5 compared to controls, suggesting an increase in stem/progenitor cells capable of initiating endometriotic lesions. However, a decrease in expression of CD146, an endometrial mesenchymal stem cell and endothelial cell marker (Schwab and Gargett, 2007; Schwab et al., 2008) was surprising and contrasted the W5C5 data (Fig. 4.11). Perhaps,

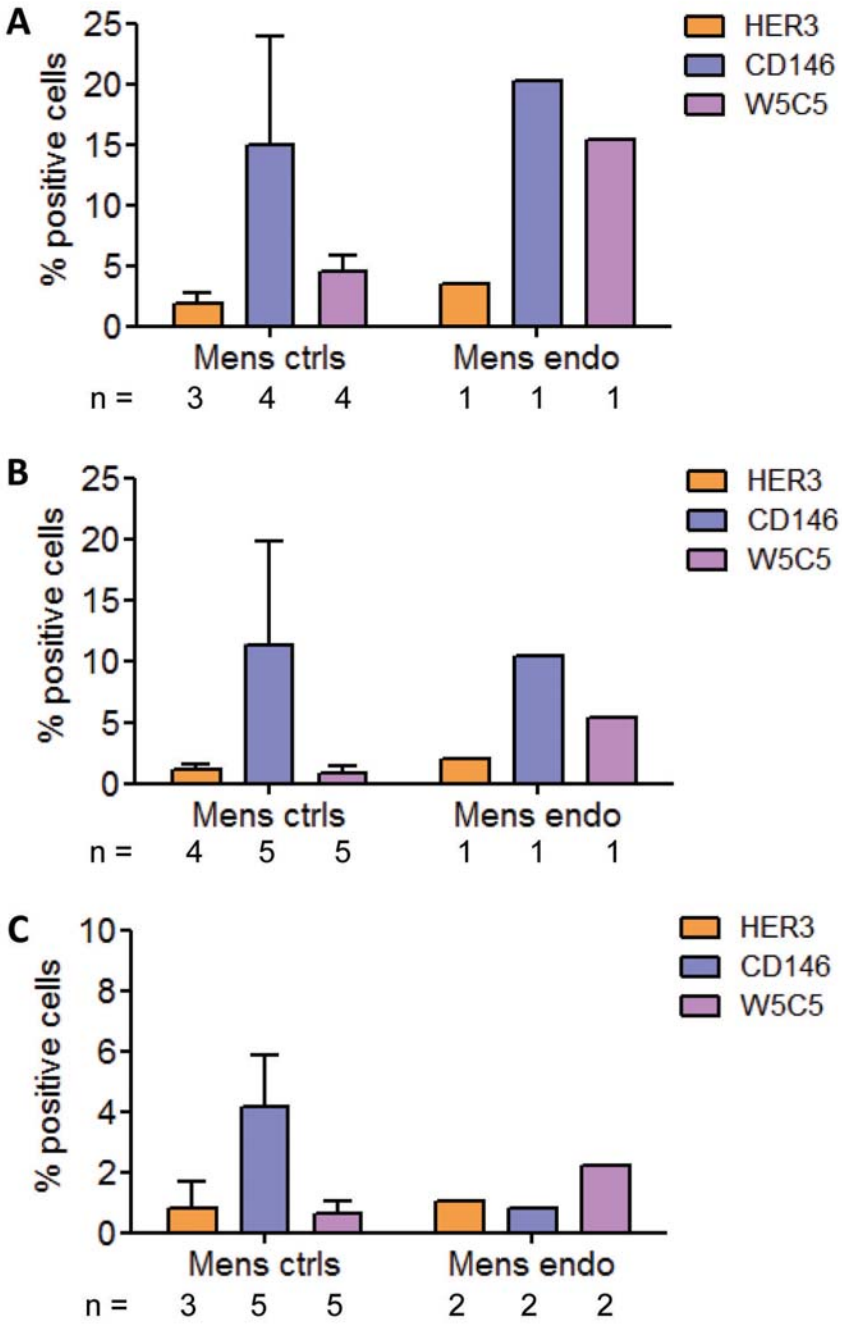


Figure 4.11: Expression of putative and known endometrial epithelial (HER3) and mesenchymal (CD146, W5C5) stem/progenitor cell markers by flow cytometry in (A) UMB epithelial-enriched cell suspensions, (B) UMB mesenchymal-enriched cell suspensions and (C) PF. Mens endo, menstruating endometriosis and mens ctrls, menstruating controls. Data represented as mean with SEM (n=2–5). NB: scale on x-axis varies.

free floating mesenchymal cells were not obtained during sample collection. However, these results may have been affected by the large variation observed in control samples, and low samples numbers of endometriosis samples. Though preliminary, the data suggest an increase of endometrial stem/progenitor cells in the pelvic cavity of menstruating endometriosis women however more samples are required to confirm this.

Endometriosis is associated with a highly inflamed intraperitoneal cavity (Haney et al., 1981; Halme et al., 1987; Loh et al., 1999). Given this, it was not surprising that the volume of PF was significantly higher in menstruating endometriosis women than controls. In future, it would be interesting to compare PF volumes in menstruating and non-menstruating women with and without endometriosis. Furthermore, markers of inflammatory cytokines, chemokines and growth factors in PF of menstruating endometriosis and control women should be compared, to identify differences which may give endometrial stem/progenitor cells in women with endometriosis a selective advantage.

Previous studies have reported similar incidences in the presence of endometrial gland-like structures in PF from both women with endometriosis and normal controls, although an *in vitro* examination of collected samples was not undertaken (Bartosik et al., 1986). Additional investigations have shown endometrial cells from PF of endometriosis and control women capable of adhering and proliferating *in vitro* following bulk culture, however no difference was identified between the two groups (Kruitwagen et al., 1991). The present study was unique in that samples were only collected from menstruating women between day 1–3 when most of the functionalis is shed (Garry et al., 2009), and cloning studies were also conducted (Chan et al., 2004; Schwab et al., 2005; Gargett et al., 2009). Seeding cells at cloning density is a stringent approach compared to bulk culturing cells as only a single adult stem/progenitor cell or TA cell can produce a colony when seeded at extremely low densities (Chan et al., 2004).

Unexpectedly, epithelial clones were only identified in one endometriosis patient's peritoneal fluid, with the remainder of the endometrial cell clones possessing mesenchymal-like

morphology. No significant difference was observed in the clonogenic activity of mesenchymal cells from the PF of endometriosis and control patients. Currently, the data does not support the hypothesis that more viable endometrial stem/progenitor cells are effluxed into the peritoneal cavity during menstruation in endometriosis patients. However, it does support the concept that endometrial stem/progenitor cells are shed into the peritoneal cavity and can therefore initiate endometriotic lesions. Data to date has not solved why endometriotic lesions establish in endometriosis women but not normal women. The addition of more patients would provide further data, which is imperative to confirm or disregard this hypothesis.

The data shown in Figure 4.4 demonstrates that five endometriotic lesions collected from two separate endometriosis patients contained clonogenic endometrial cells and provides strong support for our hypothesis that endometriotic lesions are established from endometrial stem/progenitor cells, in agreement with other reports (Kao et al., 2010). Since endometriosis is characterised by the presence of glandular tissue in ectopic lesions, the inability to identify endometrial epithelial clones in PF in the majority of patients is a limitation of this study. Endometrial epithelial cells are notoriously difficult to grow in culture, even when isolation procedures are designed for specific selection of this cell type (Musina et al., 2008). As shown in Figure 4.10, PF contained at least two to three morphologically different cell types. Thus, it was not surprising that the robust mesenchymal, and to a lesser degree mesothelial cells, were predominant on the cloning plates, and an epithelial clone was only observed on one occasion. The use of a robust epithelial marker would be valuable to separate the epithelial component of endometriotic lesions or from PF or menstrual blood, thus removing the problem of stromal and/or mesothelial overgrowth in culture. In addition, if HER3 is confirmed as a marker of endometrial epithelial progenitor cells, then the prospective isolation and characterisation of these cells in endometriosis would provide a significant breakthrough in the possible aetiology of endometriosis.

It has also been reported that endometrial cells adhere very rapidly (within 1 hr) to the mesothelial lining (Witz et al., 2002), thus few would then remain in PF at any one time. It is

plausible that the timing of PF collection was incompatible with the presence of free epithelial glands/cells and in future, continuous collection of PF could be considered, although difficult to implement in humans, it may be possible using a non-human primate model. It is also possible that endometrial epithelial cells could have transdifferentiated into mesenchymal cells *in vitro*, as demonstrated recently where human endometrial fragments were grafted into immunocompromised mice producing endometriotic lesions which were only visible after treatment with estrogen (Chen et al., 2010). Further immunohistochemical analysis of these implanted tissues revealed changes in epithelial-mesenchymal transition (EMT) markers, an increase in vimentin and decrease in E-cadherin when comparing pre-transplantation counterparts (Chen et al., 2010). Extrapolating these results to the human context, it would be interesting to investigate the role of EMT in endometriosis (Gaetje et al., 1997; Grund et al., 2008) since endometriosis is an estrogen dependent condition (Rogers et al., 2009) and ectopic endometrium has been demonstrated to have elevated levels of estrogen (Delvoux et al., 2009). Epithelial cells from eutopic endometrium from women with and without endometriosis could be investigated for EMT *in vitro*, which may explain the rare sighting of epithelial colonies in this present study. Another possibility for the lack of epithelial colonies is that the release of epithelial progenitor cells into the menstrual fluid may be a rare event that does not occur every menstrual cycle, and thus new endometriotic lesions may only be established in occasional cycles where these cells are released. This supposition would fit well with the hypothesis that endometrial epithelial cells are present in the base of glands (Gargett, 2007), an area which is retained each menstrual cycle to allow new endometrial growth and regeneration the following month. It also supports our data demonstrating the presence of only one epithelial clone identified in the samples collected from 11 patients.

From these preliminary findings, we observed no difference in the clonogencity of cells in the PF collected from endometriosis and control patients. The rapid attachment of endometrial cells to mesothelium (Witz et al., 2002; Nair et al., 2008; Kao et al., 2010) may mask the true number of endometrial stem/progenitor cells present in the peritoneal cavity as adhesion

and invasion of mesothelium could be underway during collection of samples. Further, PF collected from mild endometriosis women increases the proliferation of endometrial stromal cultures *in vitro* (Meresman et al., 1997) which could potentially accelerate attachment of free floating endometrial cells to the peritoneal wall *in vivo*. However, before any definite conclusions can be made from our data, it is essential that more patient samples are collected and analysed.

It has been reported that mesothelial cells, macrophages, leukocytes and erythrocytes are also commonly found in PF alongside endometrial cells (Haney et al., 1981; Bartosik et al., 1986; Kruitwagen et al., 1991; Bokor et al., 2009), in concordance with the heterogeneous cell populations found in this study. A panel of immunomarkers that accurately distinguished between the cell types (Fig. 4.10) showed that cells consistently expressed markers common of their cell type. Epithelial-like cells expressed pan Cytokeratin, mesenchymal-like cells expressed CD10 and CD90, and both populations were negative for CA 125 (ovarian tumor marker) (Meyer and Rustin, 2000), CD31 and α-smooth muscle actin (Gargett et al., 2009). Interestingly, previous studies have shown a large number of endometrial mesenchymal colonies express α-smooth muscle actin, suggestive of myofibroblast differentiation (Chan et al., 2004). Perhaps the microenvironment in the peritoneal cavity does not support the attachment of these types of mesenchymal cells. Mesothelial cells typically share the same immunomarkers as endometrial epithelial and mesenchymal cells (van der Linden et al., 1995; Bokor et al., 2009), which is not surprising given these three cell types are mesodermally derived (Warn et al., 2001; Herrick and Mustaers, 2004; Crowley, 2009). However, this commonality of markers did prevent the use of markers to accurately distinguish between cell types. Instead, cell types in PF collected for this study were distinguished by their distinct morphologies (Fig. 4.9), as in prior studies (Bartosik et al., 1986; Kruitwagen et al., 1991). Future studies could consider using functional markers or genes to distinguish epithelial or mesenchymal cells from mesothelial cells, as well as investigating the presence of EMT markers.

There are increasing investigations into the use of shedding menstrual blood as a readily available source of mesenchymal stem cells for cell-based therapies (Cui et al., 2007; Meng et al., 2007; Hida et al., 2008; Patel et al., 2008), although the epithelial population has been overlooked. Thus, it was anticipated that uterine and vaginal menstrual blood would contain clonogenic endometrial epithelial and mesenchymal cells, which data from this study supports. However, contrary to our hypothesis, surprisingly there was no difference in the clonogenic ability of UMB/VMB of endometriosis women compared to controls. Although further samples are required to provide a firm conclusion, the lack of difference in clonogenicity of cells obtained from the PF may indicate that our initial hypothesis that endometriosis is established because of the erroneous shedding of endometrial stem cells during menstruation cannot be substantiated given the small numbers of samples examined. It has been reported that women with endometriosis have higher volumes of refluxed menstrual blood and endometrial-tissue fragments than women without the disorder (Halme et al., 1984). This study performed *in vitro* colony-forming assays using the same cell densities between control and endometriosis groups which did not account for differences in endometrial cell counts in PF. Thus, given that endometrial stem/progenitor cells are shed in menstrual blood of most women as presented herein, an alternative hypothesis of endometriosis pathogenesis can be proposed. The increased retrograde menstrual blood in endometriosis women (Fig. 4.8) results in a larger amount of endometrial stem/progenitor cells providing greater opportunity in the development of endometriosis.

Bone marrow derived cells have been reported to differentiate into epithelial cells, albeit at a low frequency (0.01%), and engraft in the murine endometrium (Du and Taylor, 2007). They hypothesised that endometriosis may arise by differentiation of bone marrow-derived cells into ectopic endometrium (Du and Taylor, 2007). The present study found no clonogenic cells in PB *in vitro*, although cells expressing EpCAM (an epithelial marker) and CD90 (stromal marker) were detected by flow cytometry, thus the potential small contribution from bone marrow and other tissues/organs cannot be disregarded. Although it is expected and

sometimes reported that PB contains mesenchymal stem cells (Jiang et al., 2002; Du and Taylor, 2007), the absence of CFU in PB observed by this study could be due to their low numbers and indicates that the four millilitres of PB cultured in this present study were insufficient or was carried out in unsuitable culture conditions. The lack of CFU in PB cultures also suggests that the *in vivo* conditions to mobilise stem cells from the bone marrow were absent. Consequently, injury or inflammatory insults could be required to stimulate bone-marrow derived cells to transdifferentiate (Du and Taylor, 2007), and it is not known whether this phenomena occurs under physiological conditions and how often.

A major limitation of this study is the small numbers of patients investigated. It has been challenging to recruit patients who are willing to track menstrual patterns over 2–3 months and to then schedule them for surgery during set operating days of the gynaecologist working with us, the hospital, and access to equipment. Even more challenging is the selection of patients at day 1–3 who also have endometriosis. Although strict, this criteria was necessary as UMB and PF samples collected from day 4 menstruating controls (n=2, data not shown) contained few endometrial fragments that did not form colonies, which is not surprising considering most endometrium is shed during the first three days of menstruation (Ludwig and Spornitz, 1991; Garry et al., 2009). It would also be ideal to continue collecting matched samples of PF and PB from endometriosis women during menstruation and non-menstruation for comparisons of clonogenicity. This present study collected samples from one endometriosis patient during menstruation and non-menstruation however results are not shown as more patients are needed. For the purpose of this thesis, all endometriosis data was combined regardless of disease severity as categorising into stages would have meant insufficient numbers for statistical analysis. The ongoing collection of samples from endometriosis women would allow investigation of a relationship between PF clonogenicity and endometriosis severity in future. Another potential confounding factor of this study is that recruited control women were usually undergoing diagnostic laparoscopy and potentially not free from underlying gynaecological problems. Thus it would have been preferable to only recruit women who

were undergoing tubal ligations as controls, however time constraints did not allow this.

This study is the first to investigate endometrial stem/progenitor cells in shedding endometrium and PF of menstruating endometriosis women and controls. Clonogenic endometrial cells were identified during the first three menstrual days with no differences observed between the two groups on the small number of samples examined. The number of cells expressing putative endometrial stem/progenitor cell markers was apparently greater in endometriosis women. In future a greater number of samples need to be studied before a more definitive conclusion can be made. Nevertheless, this studyâĂŹs observations are consistent with a previous report that endometrial tissue in PF was seen as commonly in endometriosis and control women (Bartosik et al., 1986). It is also consistent with the observation that most women have the potential to develop endometriosis but the condition develops only in those with predisposing factors. These factors could be due to varying levels of macrophages and cytokines present in peritoneal fluid (Halme et al., 1984, 1987; Kyama et al., 2006; Hever et al., 2007; Cosín et al., 2010) or predisposition to mesenchymal-epithelial transition (Chen et al., 2010), however results are conflicting (Malik et al., 2006; Hassa et al., 2009) and more investigations are required. Despite the lack of data supporting our original hypothesis, this study demonstrates importantly for the first time the presence of endometrial stem/progenitor cells in the PF of menstruating women, and also provides a platform for future investigations comparing the intraperitoneal environment of women with endometriosis to those without.

4.5 Acknowledgements

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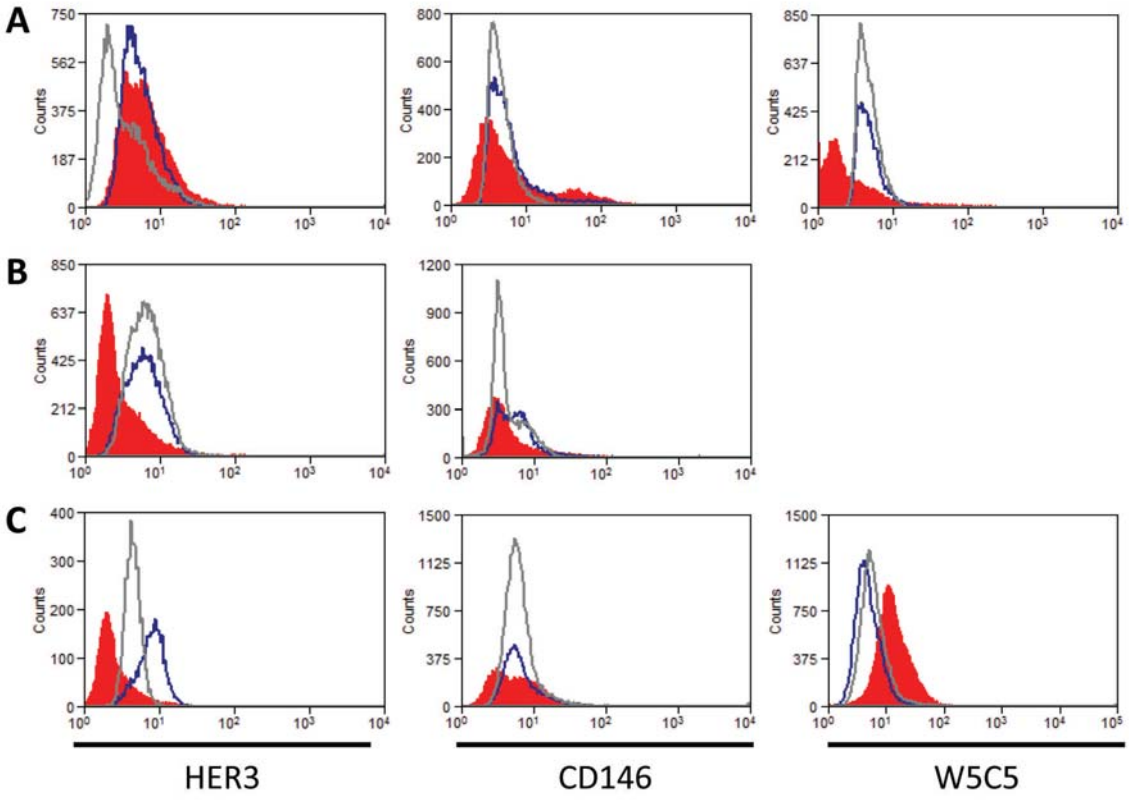

Supplementary Figure 4.12: Expression of endometrial stem/progenitor cell markers by flow cytometry in shedding endometrium from the uterine cavity (A) epithelial enriched fraction, (B) mesenchymal enriched fraction and (C) peritoneal fluid. Data represented as single parameter flow cytometry histograms. Grey line, isotype control; blue line, menstruating controls and red area, menstruating endometriosis.

		Epithelial		Mesenchymal			Mesothelial	Leukocytes	Endothelial
		HER3	**EpCAM**	**CD90**	**CD146**	**W5C5**	**Mesothelin**	**CD45**	**CD31**
PF	Mens endo	1.04 (2)	0.17 (1)	0 (1)	0.83 (2)	2.24 (2)	0.56 (1)	55.43 (1)	N/D
	Mens ctrls	0.83 ± 0.83 (3)	8.68 ± 3.47 (3)	2.16 (2)	4.17 ± 1.72 (5)	0.61 ± 0.44 (5)	0.88 ± 0.88 (4)	26.3 (2)	N/D
	NM endo	0.03 (2)	0.42 (2)	0.43 (2)	0 (2)	1.1 (2)	0 (2)	69.91 (1)	26.18 (1)
	NM ctrls	0.02 ± 0.02 (3)	0.48 ± 0.26 (3)	0.12 ± 0.08 (3)	0.23 ± 0.12 (3)	0.51 ± 0.26 (3)	0.003 ± 0.003 (3)	94.71 (1)	30.98 (1)
UMB -E	Mens endo	3.47 (1)	N/D	N/D	20.2 (1)	15.43 (1)	N/D	N/D	N/D
	Mens ctrls	1.81 ± 0.92 (3)	12.48 ± 2.74 (3)	49.6 (1)	14.92 ± 9.02 (4)	4.48 ± 1.35 (4)	0.04 ± 0.04 (3)	19.35 (2)	N/D
UMB -S	Mens endo	1.97 (1)	N/D	N/D	10.36 (1)	5.41 (1)	N/D	N/D	N/D
	Mens ctrls	1.06 ± 0.46 (4)	7.9 ± 3.76 (4)	18.21 (2)	11.34 ±8.4 (5)	0.85 ± 0.58 (5)	2.23 ± 2.17 (4)	24.45 (2)	N/D
VMB - E	Mens ctrl	0 (1)	15.1 (2)		13.35 (2)	3.26 (2)	2.08 (2)	24.73 (2)	N/D
VMB - S	Mens ctrl	0 (1)	20.09 (2)	N/D	23.14 (2)	8.63 (2)	0.16 (2)	28.45 (2)	N/D
PB	Mens endo	0 (1)	0.32 (1)	0.21 (1)	0 (1)	0 (1)	0 (1)	71.6 (1)	N/D
	Mens ctrl	0 (3)	3.71 ± 3.71 (3)	0 (1)	0 (4)	0 (4)	0.26 ± 0.26 (3)	N/D	N/D
	NM endo	0 (1)	0.2 (1)	0.33 (1)	0 (1)	0 (1)	0 (1)	57.6 (1)	N/D
	NM ctrl	0 (3)	0.04 ± 0.02 (3)	0.17 ± 0.17 (3)	N/D	0.03 ± 0.03 (3)	0 (3)	30.0 (2)	2.47 (1)

Supplementary Table 4.13: Samples collected from menstruating women with and without endometriosis were profiled by flow cytometry. Data reported as mean ± SEM % positive cells (number of samples). PF, peritoneal fluid; UMB-E, uterine menstrual blood-epithelial fraction; UMB-S, uterine menstrual blood-stromal fraction; VMB-E, vaginal menstrual blood-epithelial fraction; VMB-S, vaginal menstrual blood-stromal fraction; PB, peripheral blood. Mens endo, menstruating endometriosis; Mens ctrls, menstruating controls; NM endo, non-menstruating endometriosis; NM ctrls, non-menstruating controls. N/D, not done.

CHAPTER 5

General Discussion

This is first study to have identified a putative marker for the prospective isolation of human endometrial epithelial progenitor cells. In the absence of any known marker for endometrial epithelial progenitor cells, a strategy was devised to screen endometrial epithelial cells with a panel of novel and known surface markers designed to identify adult stem cells (Chapter 2). This systematic approach using flow cytometry and immunohistochemistry yielded a potential marker, HER3, which was shown to enrich for endometrial epithelial progenitors in a limited number of patient samples analysed (Chapter 3).

Without a marker, human endometrial epithelial progenitors cannot be fully characterised, as the functional assays used to previously identify this population were retrospective. The identification of a marker allowed investigations into the potential role of human endometrial epithelial progenitors in the pathogenesis of gynaecological disorders such as endometriosis. The utility of HER3 as a marker was evaluated using *in vitro* functional assays for clonogenic endometrial epithelial cells in peritoneal fluid and shedding endometrium from menstruating women with and without endometriosis (Chapter 4).

The purpose of this last chapter is to discuss the implications of identifying a putative marker for endometrial epithelial progenitors, the presence of clonogenic endometrial cells in endometriosis and control women, and lastly to identify future directions that build upon the

findings of this project.

5.1 Identifying candidate markers of human endometrial epithelial progenitor cells

Since there were no known markers of endometrial epithelial progenitor cells prior to this project, the search commenced by assessing novel and known surface markers for expression in endometrium. Six antibodies (from a total of 24) formed a priority list of candidate endometrial epithelial progenitor markers. Of the six candidate markers, HER3 was chosen as top priority given its stronger expression in basalis endometrium, heterogeneous staining of glandular epithelia, glandular expression in the inactive endometrium and its epitope withstood enzymatic tissue dissociation. In well characterised epithelial stem cell systems such as the mammary gland (Stingl et al., 1998, 2001), a combination of markers was used to prospectively isolate epithelial stem cells. Applying this to the endometrium, a single marker such as HER3 is most likely to only partially purify endometrial epithelial progenitor cells *in vivo*, as demonstrated by the only two- and four-fold increase in clonogenic cells observed for the $HER3^{+}EpCAM^{-/+}$ subpopulations. The remaining five candidate markers should be investigated as potential co-markers for use with HER3 for the purpose of improving the enrichment of endometrial epithelial progenitor cells. Alternatively, a genomic approach could be used to identify more markers through gene expression profiling to compare the freshly isolated HER3 enriched epithelial progenitor population to a HER3 depleted epithelial population. In the mammary gland, this approach identified genes specific for undifferentiated cells involved in the maintenance of an undifferentiated state and self-renewal (Dontu et al., 2003; Behbod et al., 2006). Similarly, microarray studies of human endometrial side population (SP) epithelial cells have also identified specific genes (Cervello et al., 2010), however further work is needed to assess the potential of these genes as markers

for enriching epithelial progenitor cells. Furthermore, transcriptional profiling will allow for elucidation of the signaling pathways that govern self-renewal and differentiation leading to a better understanding of the role epithelial progenitor cells may play in endometrial diseases.

5.2 HER3 is a putative marker of endometrial epithelial progenitor cells

This study (Chapter 3) assessed the utility of HER3 as a marker of human endometrial epithelial progenitor cells using functional assays of stem/progenitor cell activity. For the first time, HER3 in combination with EpCAM enriched for a small population of endometrial epithelial cells capable of colony formation and self-renewal.

In order to prospectively identify epithelial progenitors using markers, the approach used in this project was based upon those used in the mammary gland and prostate (Stingl et al., 2001; Lawson et al., 2007). The mammary gland and prostate are tissues comparable to the endometrium as all are regulated by sex steroids and undergo cellular changes in response to fluctuating levels of sex hormones. However, in comparison to the endometrium, the mammary gland and prostate are relatively well characterised. Several markers have been used in different combinations to form a defined set that distinguish mammary and prostate progenitor cells from their mature progeny (Richardson et al., 2004; Shackleton et al., 2006; Stingl et al., 2006; Lawson et al., 2007; Lim et al., 2009). Of these previously used markers, CD133 was of particular interest to this study as it has been used to identify adult stem cells in several other tissues (Handgretinger et al., 2003; Marzesco et al., 2005; Oshima et al., 2007; Yovchev et al., 2007) and cancer stem cells (Richardson et al., 2004; O'Brien et al., 2007; Vander Griend et al., 2008). Initially, this thesis (Chapter 2) investigated CD133 as a potential epithelial progenitor marker, however CD133 immunostained the entire luminal and glandular epithelia. The expression of a suitable marker of rare progenitor cells is not expected to appear

in high numbers throughout the endometrium, thus CD133 was not pursued.

Prior to this study, the lack of surface markers for the isolation of endometrial epithelial progenitors was a major impediment to the characterisation of these cells. Investigators instead have relied upon the *Brcp1/ABCG2* gene to mark SP cells which are a heterogeneous population of endometrial stem/progenitor cells (Kato et al., 2007; Tsuji et al., 2008; Cervello et al., 2010; Masuda et al., 2010). Reports on the percentage of endometrial epithelial SP have varied between 0.01–6.2% (Kato et al., 2007; Cervello et al., 2010; Masuda et al., 2010), compared to 0.22–0.52% of epithelial progenitors identified through functional assays (Chan et al., 2004). This study found 0.4% and 0.8% of epithelial progenitors in the $HER3^{+}EpCAM^{+}$ and $HER3^{+}EpCAM^{-}$ subpopulations respectively. Although SP studies have reported a higher purification of endometrial epithelial progenitors, this enrichment was not pure and predominantly contained endothelial cells and contaminating stromal cells (Tsuji et al., 2008; Cervello et al., 2010). The SP assay is not without its limitations as it requires use of a DNA intercalating dye (Hoechst 33342) which has been associated with significant cellular toxicity and is technically difficult (Welm et al., 2002; Montanaro et al., 2004; Tadjali et al., 2006). Furthermore, whilst the SP assay identifies stem cells, it does not account for all stem cells of a tissue at any one time (Morita et al., 2006). The limitations of the SP assay, in particular cellular toxicity do not make it a suitable method for prospectively identifying epithelial cells and for this reason was not a technique used in this thesis.

Accumulating published evidence and this study support the presence of epithelial progenitor cells in the endometrium (Chan et al., 2004; Schwab et al., 2005; Gargett et al., 2009; Masuda et al., 2010). However, the origin of the endometrial stem cell niche remains to be determined. Morphological studies suggest that the endometrium does not regenerate from remaining basal epithelial glands following menstruation but rather neighbouring stroma (Baggish et al., 1967; Garry et al., 2009, 2010). The findings of this study do not support this. Expression of HER3 was specific for endometrial epithelia whose expression was stronger in the basal glands of cycling and inactive endometrium and is supported by studies of mouse

endometrium suggesting that one endometrial stem/progenitor cell niche is located in the basal glands (Kaitu'u-Lino et al., 2010). In order to elucidate the location of the niche, HER3 gene expression could be compared in glands laser-captured from basalis and functionalis across the cycle and from post-menopausal endometrium, and the expression of stem cell related genes and those associated with their properties compared. Alternatively, populations of basalis and functionalis epithelial cells should be isolated and flow cytometry sorted for comparison using functional stem cell assays *in vitro*, or transplantation into murine models. It would be expected that the basalis epithelial population would contain more clonogenic and self-renewing cells than functionalis epithelial cells, and thus should provide more information regarding the location of the epithelial stem cell niche. However, a robust marker differentiating basalis and functionalis glands is required, such as CD203c identified in Chapter 2, a potential marker that may expedite locating the endometrial epithelial progenitor cell niche.

This thesis has provided the first data in identifying and assessing a putative marker, however further confirmatory studies are necessary. The development of a three-dimensional culture system for the *in vitro* differentiation of an epithelial progenitor is crucial to better understand these rare cells, as the microenvironment formed simulates an *in vivo* system (Härmä et al., 2010). Thus, a three-dimensional culture system is better suited to address questions of cell biology than a traditional two-dimensional monolayer (Härmä et al., 2010). In the mammary gland, the development of a three-dimensional Matrigel culture system has supported the differentiation of mammary epithelial stem cells into functional mature progeny in the presence of inductive medium (Lim et al., 2009). A similar model of three-dimensional culture is being developed for clonally derived endometrial epithelial cells (Gargett et al., 2009) which should be used to validate HER3 as a marker of epithelial progenitor cells, as well as any other potential markers in the future. However, the highest level of stem cell assessment requires a putative stem/progenitor cell to reconstitute tissue *in vivo*. Studies have demonstrated that unfractionated endometrial cells transplanted into immunocompromised mice were able to produce endometrial-like tissue that was responsive

to estrogen and progesterone, and underwent menstruation (Kurita et al., 2001; Masuda et al., 2007). To validate HER3 as a marker that partially purifies endometrial epithelial progenitors, sorted HER3/EpCAM subpopulations with supporting stroma should be xenotransplanted into murine models and examined for the *in vivo* reconstitution of endometrial tissue. In addition, as other markers for epithelial progenitors are discovered, they too should be xenotransplanted into murine models to assess their ability to reconstitute endometrial tissue.

The identification of HER3 as a surface marker that partially purifies epithelial progenitor cells from their mature progeny is the first step towards identifying more markers that can be co-utilized to enrich for endometrial epithelial progenitor cells. This is an exciting discovery as without such markers, the full characterisation of this population would be severely hampered. While this is early evidence, it provides a major breakthrough to enable future investigations into the role of epithelial progenitor cells in gynaecological disease.

5.3 Endometriosis

The hypothesis that endometrial stem/progenitor cells are refluxed during menstruation into the peritoneal cavity in women who develop endometriosis is an attractive postulate that explains why only some women develop endometriosis even though most women experience retrograde menstruation. In order to support or refute this, Chapter 4 investigated shedding eutopic endometrium and peritoneal fluid from menstruating women with and without endometriosis for the presence of endometrial stem/progenitor cells. Samples collected from patients were examined for HER3 expression, demonstrating the usefulness of an epithelial progenitor cell marker in broadening the investigations possible for studying endometrial disorders.

The presence of endometrial cells has been reported in peritoneal fluid of women with and without endometriosis, however none of these studies investigated the stem cell properties of these cells (Bartosik et al., 1986; Kruitwagen et al., 1991; Bokor et al., 2009). Therefore, the most significant finding of this study demonstrated that endometrial stem/progenitor cells

are shed during menstruation, as clonogenic cells were obtained from shedding endometrium and were present in peritoneal fluid. However, the hypothesis that there is a difference in the number of clonogenic cells within shedding endometrium collected from women with and without endometriosis was not supported by the data, albeit very preliminary. Interestingly, flow cytometry data on fresh samples from endometriosis women revealed an increased number of cells expressing putative endometrial stem/progenitor cell markers (HER3 and W5C5), suggesting that the sample size needs to be increased before any definite conclusions can be made.

This study also supports that retrograde efflux of menstruation occurs, but the lack of difference in the number of clonogenic cells between endometriosis and control women suggests that retrograde menstruation alone is not responsible for the development of endometriosis. Instead, two hypotheses are proposed. The first hypothesises that factors present in peritoneal fluid support the growth of ectopic endometrial stem/progenitor cells which results in the development of endometriosis (Fig 5.1A). The second hypothesises that factors present in peritoneal fluid support an interaction between endometrial stem/progenitor cells and free-floating peritoneal mesothelial cells which results in mesothelial cells undergoing an epithelial-mesenchymal and/or a mesenchymal-epithelial transition which results in mesothelial cells acquiring an invasive cellular phenotype and leads to the development of endometriosis in some women (Fig 5.1B).

Reports have demonstrated a potential role for macrophages, cytokines and chemokines present in peritoneal fluid in supporting the growth of ectopic endometrium (Halme et al., 1984, 1987; Kyama et al., 2006; Hever et al., 2007; Cosín et al., 2010). Proliferation of eutopic endometrial stromal cells increased in the presence of peritoneal fluid from women with endometriosis, suggesting that peritoneal fluid contains mitogenic factors, although the exact mechanisms remain unknown (Surrey and Halme, 1990; Meresman et al., 1997; Braun et al., 2002). Therefore, the mitogenic effects of peritoneal fluid on eutopic and ectopic endometrial stem/progenitor cells should be determined, which may reveal that

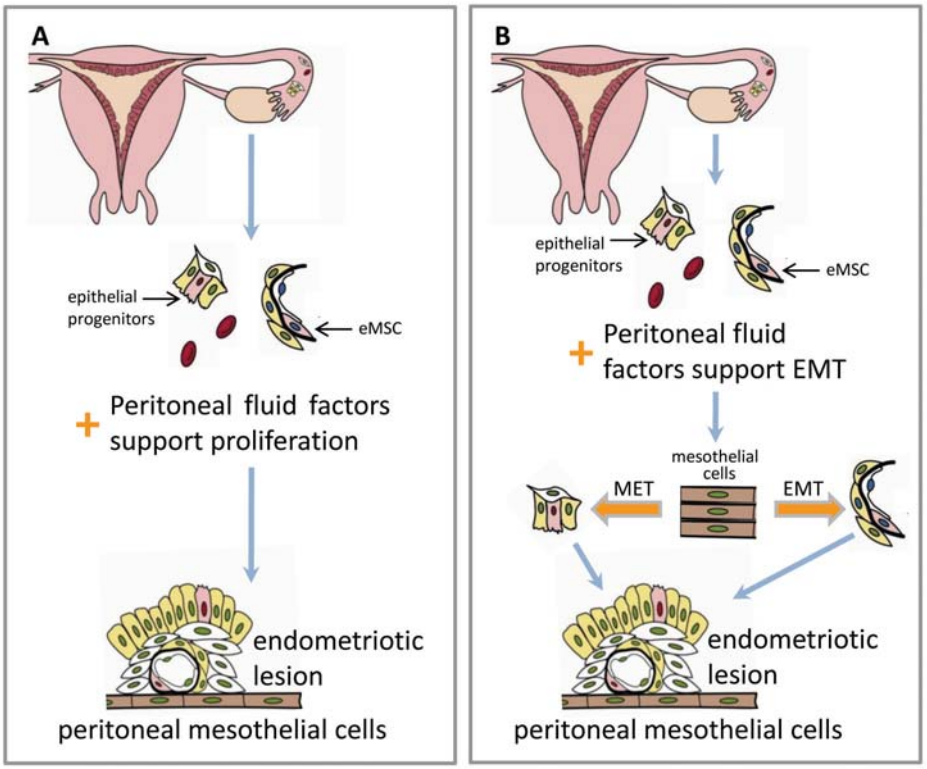

Figure 5.1: Possible role for factors contained within peritoneal fluid in the pathogenesis of endometriosis. Endometrial stem/progenitor cells are effluxed into the peritoneal cavity during menstruation (A) where factors in the peritoneal fluid support their proliferation. Subsequent adhesion, proliferation and establishment of endometriotic lesions ensues. Or (B) where factors in the peritoneal fluid interact with eutopic endometrial stem/progenitor cells and also induce free-floating peritoneal mesothelial cells to undergo an epithelial-mesenchymal transition (EMT) and/or mesenchymal-epithelial transition (MET) which results in the establishment of endometriotic lesions. eMSC, endometrial mesenchymal stem cell. Panel A is adapted with permission from Gargett and Masuda (2010) as published in *Mol Hum Reprod* 2010, 16(11):818-34.

factors in peritoneal fluid and their interaction with endometrial stem/progenitor cells are responsible for the development of endometriosis rather than the mere presence of endometrial stem/progenitor cells in the peritoneal cavity. Alternatively, ectopic endometrial cells may induce an inflammatory response in the peritoneal cavity, releasing chemoattractants that result in an uncharacteristic influx of inflammatory cells (Song et al., 2003), thus creating conditions more conducive to the initiation of endometriotic lesions. The *in vitro* proliferation of endometrial stem/progenitor cells supplemented with inflammatory molecules likely present in the peritoneal cavity should also be investigated in future.

Mesothelial cells line the cavity of the peritoneum and reportedly undergo rapid repair (within 24 hours) following injury (Mutsaers et al., 2002), similar to the rapid repair of human endometrial epithelium (McLennan and Rydell, 1965; Ludwig and Spornitz, 1991; Kaitu'u-Lino et al., 2007; Garry et al., 2009). Emerging reports suggest that free-floating mesothelial progenitor cells (Foley-Comer et al., 2002) are involved in the repair of the peritoneum (Warn et al., 2001; Herrick and Mustaers, 2004) and are plastic, undergoing differentiation into smooth muscle (Pampinella et al., 1996). Sharing the same mesodermal heritage as endometrial epithelial and mesenchymal cells, mesothelial cells also exhibit characteristics of epithelial and mesenchymal phenotypes (van der Linden et al., 1995; Warn et al., 2001; Herrick and Mustaers, 2004; Bokor et al., 2009). In this study it was observed that peritoneal fluid contained clonogenic cells that were clearly not an endometrial phenotype, but were morphologically mesothelial. Given the presence of these mesothelial cells in peritoneal fluid and accumulating data from models of endometriosis suggesting that mesothelial cells can undergo an epithelial-mesenchymal transition (Demir Weusten et al., 2000; Demir et al., 2004; Nair et al., 2008), and from studies of human ovarian surface epithelium (mesothelial derivative) suggesting the mesenchymal-epithelial transition (Auersperg et al., 1999; Bendoraite et al., 2010), future studies should investigate the role of mesothelial cells and endometrial mesenchymal stem cells and epithelial progenitor cells in the formation of endometriotic lesions. To investigate the likelihood of this phenomenon occurring, co-cultures of mesothelial

and endometrial mesenchymal stem cells or epithelial progenitor cells could be compared to co-cultures of endometrial mesenchymal stem cells and epithelial progenitor cells in the presence of peritoneal fluid collected from menstruating endometriosis women for clonogenic activity and possible epithelial-mesenchymal or mesenchymal-epithelial transitions. In addition, peritoneal fluid from control women should also be compared to determine the normal interaction between mesothelial cells and peritoneal fluid. Alternatively, mesothelial cells could be co-transplanted with endometrial stem/progenitor cells into murine models for investigating the *in vivo* reconstitution of endometriotic tissue.

Endometriosis is a multifactorial disease and given the estimated 51% heredity (Painter et al., 2011) suggests a complex interplay between effluxed viable endometrial fragments and genetic components may lead to the development of endometriosis. Recently, genome wide studies have identified *Wnt4*, a gene involved in regulating proliferation of the endometrium (Bui et al., 1997) was related to an increased risk of endometriosis (Painter et al., 2011). These findings indicate a possible fundamental difference in the signalling pathway of endometrial stem/progenitor cells in women with and without endometriosis (Painter et al., 2011). Potentially, these pathway differences could affect the regulation and control of the endometrial stem/progenitor cells effluxed in the peritoneal cavity and initiation of disease occurs, however understanding of these mechanisms will require functional assessments. An emerging area of research in elucidating the mechanisms involved in the development of endometriosis are microRNAs (miRNAs), potential master regulators of cellular processes (Ohlson Teague et al., 2009; Teague et al., 2010). Microarray studies have identified the upregulation of two supressor miRNAs (miR-125a and miR-125b) that repress HER3 in ectopic lesions, in comparisons between paired samples of eutopic and peritoneal ectopic endometrial tissue (Teague et al., 2010) which interestingly is in conflict with the present study's preliminary data. This disconcordance could be contributed by differences in sample collection and severity of endometriosis samples. The present study compared cells recently shed into the peritoneal fluid whilst Teague et al. (2010) examined well established endometriotic lesions, where

the contribution of endometrial stem/progenitor cells is potentially minimal compared to individual free-floating cells. Also, the present study obtained samples from two women both with stage one endometriosis whilst Teague et al. (2010) combined ectopic endometrial tissue with the severity of individual paients unknown. The expression of HER3 could potentially be increased during early stages but down-regulated in severe endometriosis, however more samples are required for a conclusion to be made on HER3 expression in endometriosis.

For the first time, preliminary data from this study has demonstrated the presence of endometrial clonogenic cells in peritoneal fluid of women with and without endometriosis. Further examination is required to determine if these ectopic endometrial cells fulfill the definition of adult stem cells by demonstrating functional properties of self-renewal, high proliferative potential and ability to differentiate *in vitro* and *in vivo* into functional cells. Results of this early study seemingly refute the hypothesis that the efflux of viable endometrial stem/progenitor cells leading to endometriosis only occurs in women with endometriosis but rather is a general phenomenon. However, not all clonogenic cells are stem cells and since the clonogenic cells from peritoneal fluid have not yet been fully characterised, the numbers of CFU that self-renew, differentiate and have high proliferative potential may yet be different between women with and without endometriosis. Therefore, further investigations must be carried out before disregarding this hypothesis. In addition, future investigations could also examine the factors present in peritoneal fluid and their interactions with endometrial stem/progenitor cells and mesothelial cells for their role in the development of endometriosis.

5.4 Endometrial epithelial progenitor cells and endometrial cancer

Accumulating evidence supports the role of cancer stem cells in the initiation and propagation of carcinogenesis. Cancer stem cells (CSC) are thought to have acquired step-wise mutations

which enable unregulated self-renewal and proliferation (Reya et al., 2001; Visvader and Lindeman, 2008; Hubbard and Gargett, 2010). Many parallels have been drawn between CSC and normal stem cells (Reya et al., 2001; Pardal et al., 2003). HER3 expression has been linked with many types of cancer, including breast, ovarian and prostate (Mellinghoff et al., 2004; Agus et al., 2005; Tanner et al., 2006). The ability of HER3 to bind HER2, creating a HER2/HER3 heterodimer co-receptor, creates a potent mitogen and transformation signal (Mellinghoff et al., 2004). Thus, this heterodimer has been linked with the development and progression of human prostate cancer through the activation of both mitogen activated kinase (MAPK) and phosphatidylinositol-3 kinase (PI-3K) pathways (Mellinghoff et al., 2004; Gregory et al., 2005). Hence, we postulate that endometrial cancer may be derived from an increased number of $HER3^{+}$ endometrial epithelial progenitors amplified through acquired mutations and epigenetic changes that contribute to CSC phenotype. Future experiments could investigate whether HER3 isolates tumour-initiating, clonogenic and self-renewing endometrial cancer cells by comparing $HER3^{+}$ cells in benign and malignant endometrium and subjecting them to *in vitro* and *in vivo* cancer stem cell assays (Hubbard et al., 2009).

5.5 Conclusion

In the context of adult stem cells, the endometrium is under-recognised as a highly regenerative tissue. A better understanding of the role of endometrial epithelial progenitor cells in the processes of growth and differentiation of the endometrium holds much promise in gaining more insight into normal endometrial biology, and how aberrations in their regulation and maintenance may result in the development of gynaecological disease. This could lead to the development of better therapies, especially those that may specifically target these potentially disease-initiating cells and hopefully provide a permanent cure rather than merely treating recurrent symptoms, as is currently the case for endometriosis. In order to achieve this, identification of surface markers is required to characterise this rare population.

This is the first study to identify a marker that partially purifies endometrial epithelial progenitor cells. The findings of this study provide a foundation for future investigations into the molecular, genetic and cellular characteristics of endometrial epithelial progenitors and the regulatory signaling pathways that govern their function in maintaining tissue homeostasis. Also, the location of the endometrial epithelial stem/progenitor cell niche, the further identification of more specific markers and their possible role in endometrial proliferative disorders could be examined. Indeed, this study applied the HER3 marker to investigate the shedding of endometrial stem/progenitor cells in endometriosis. It is also the first study to demonstrate the presence of candidate endometrial stem/progenitor cells in the peritoneal fluid of menstruating women and in endometriotic lesions, supporting the dogma that retrograde menstruation contributes to endometriosis initiation and serves to validate that epithelial progenitors reside in the endometrium.

Appendix

STEM CELLS AND DEVELOPMENT
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The Mesenchymal Stem Cell Antigen MSCA-1 is Identical to Tissue Non-specific Alkaline Phosphatase

Malgorzata Sobiesiak,[1] Kavitha Sivasubramaniyan,[1] Clemens Hermann,[2] Charmaine Tan,[3] Melanie Örgel,[1] Sabrina Treml,[1] Flavianna Cerabona,[1] Peter de Zwart,[4] Uwe Ochs,[4] Claudia A. Müller,[5] Caroline E. Gargett,[3] Hubert Kalbacher,[2] and Hans-Jörg Bühring[1]

We have recently identified 2 distinct CD271^{bright}MSCA-1^{dim}CD56^{+} and CD271^{bright}MSCA-1^{bright}CD56^{-} MSC subsets in primary femur-derived bone marrow (BM), which differ in their expression pattern and morphology as well as in their clonogenic and differentiation capacity. Here, we show that MSCA-1 is identical to tissue non-specific alkaline phosphatase (TNAP), an ectoenzyme known to be expressed at high levels in liver, bone, and kidney as well as in embryonic stem (ES) cells. SDS-PAGE of WERI-RB-1 cell lysate and supernatant from phosphatidylinositol-specific phospholipase C (PI-PLC)-treated WERI-RB-1 cells resulted in the appearance of a prominent 68-kDa band. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) sequence analysis revealed TNAP-specific peptides. Screening of the MSCA-1-specific antibody W8B2 on HEK-293 cells transfected with the full-length coding sequence of TNAP showed specific reactivity with transfected but not with parent cell line. In addition, TNAP-specific mRNA expression was selectively detected in the transfectant line. In agreement with these findings, enzymatic activity of TNAP was exclusively detected in sorted MSCA-1^{+} BM cells but not in the MSCA-1^{-} negative fraction. Surface marker analysis revealed coexpression of the embryonic marker SSEA-3 but not SSEA-4, TRA-1-60, and TRA-1-81. In endometrium, TNAP is expressed at intermediate levels on CD146^{+} cells and at high levels in the luminal space of glandular epithelia. Our results demonstrate that TNAP is a selective marker for the prospective isolation of BM-derived MSC and MSC-like cells in endometrium.

Introduction

Mesenchymal stem/stromal cells (MSC) can give rise to mesodermally derived tissues including bone, cartilage, muscle, stromal cells, tendon, and connective tissue [1–5]. In culture, they adhere to the plastic surface where they display a fibroblastoid shape. Cultured bone marrow (BM)-derived MSC express a panel of key markers including CD29, CD44, CD73, CD90, CD105, CD166, CD271, CD349, and mesenchymal stem cell antigen-1 (MSCA-1; W8B2 antigen) but lack expression of CD45, CD34, and CD133 [1,6,7].

In common protocols, unfractionated bone marrow-derived cells are used as the starting population for the culture of MSC. This isolation method is based on the adherence of fibroblast-like cells to the plastic surface and the removal of nonadherent hematopoietic cells [1,2,4,8]. The resulting cells are poorly defined and give rise not only to heterogeneous MSC populations but also to osteoblasts and/or osteoprogenitor cells, fat cells, reticular cells, macrophages, and endothelial cells [9,10]. To define the starting population more precisely, surface markers such as SSEA-4, the ganglioside GD2, CD140b, CD146, CD200, CD271, the αv/β5 integrin complex, as well as several antibody-defined molecules were used for the prospective isolation of MSC [11–17]. The latest markers include MSCA-1 and CD56, which have been proved to be suitable surface molecules to purify and characterize MSC subsets from primary bone marrow [18].

Tissue non-specific alkaline phosphatase (TNAP) belongs to a large family of dimeric enzymes common to

[1]Department of Internal Medicine II, Division of Hematology, Immunology, Oncology and Rheumatology, [5]Center for Medical Research, Section for Transplantation Immunology and Immunohematology, University Clinic of Tübingen, Tübingen, Germany.
[2]Interfaculty Institute of Biochemistry, [4]BG-Trauma-Center, Department of Traumatology, Division of Arthroplasty, University of Tübingen, Tübingen, Germany.
[3]Centre for Women's Health Research, Monash Institute of Medical Research and Monash University Department of Obstetrics and Gynaecology, Monash Medical Centre, Melbourne (VIC), Australia.

all organisms. They catalyze the hydrolysis of phosphomonoesters with release of inorganic phosphate (Pi) [19,20]. TNAP is an ectoenzyme anchored to the membrane via a covalent linkage to phosphatidylinositol (PI) that can be cleaved by bacterial PI phospholipase C (PI-PLC) [21,22]. Four isoforms of this enzyme family are described including intestinal, placental, placenta-like, and tissue non-specific phosphatases [19,20]. TNAP is present in bone, liver, kidney, and endometrium [23–26] and is expressed at high levels on the surface of embryonal carcinoma (EC) and embryonic stem (ES) cells [27]. During differentiation of ES cells, the expression level of TNAP decreases. In the present work, we describe that MSCA-1 is identical to TNAP. In addition, we show differential expression of this ectoenzyme in $CD56^+$ and $CD56^-$ bone marrow MSC subsets in the hip joint, as well as in glandular epithelia and MSC-like cells of the endometrium.

Materials and Methods

Primary cells and tissues

Bone marrow cells. Bone marrow from femur, acetabulum, trochanter, and trabeculum was harvested at the Hospital for Workers Compensation from the femoral shafts of patients undergoing total hip replacement. Cells were collected in 5,000 U heparin (Sigma-Aldrich, Taufkirchen, Germany) after informed consent and approval of the Ethics Committee of the University of Tübingen. Bone marrow mononuclear cells were isolated by Ficoll Histopac density gradient fractionation and remaining erythrocytes were lysed in ammonium chloride solution.

Endometrium. Endometrial tissues ($n = 11$) were collected from women (aged 42.7 ± 4.6 years [±SD], range 36–52 years) undergoing hysterectomy, who had not received hormonal treatment 3 months prior to surgery. Ethics approval was obtained from the Southern Health Human Research and Ethics Committee C and informed written consent was obtained from each patient. Menstrual phase was assessed by histological examination according to well-established criteria; proliferative ($n = 4$), secretory ($n = 5$), weakly proliferative ($n = 1$), and inactive stage ($n = 2$). Full thickness endometrium with 5 mm myometrium was collected in medium containing HEPES-buffered Dulbecco's modified Eagle's medium/Hams F-12 (DMEM/F12; Invitrogen, Carlsbad, CA), 5% newborn calf serum (CSL, Parkville, Australia), and 1% antibiotic antimycotic solution (final concentrations: 100 mg/mL penicillin G sodium, 100 mg/mL streptomycin sulfate, 0.25 mg/mL amphotericin B; Invitrogen) and processed within 2–24 h, or frozen in optimum cutting temperature (OCT) Tissue Tek® (Sakura Finetek Co., Tokyo, Japan) on dry ice and stored at −80°C until required.

Isolation of $W8B2^+$ cells by MACS and FACS

In selected experiments, the cells were pre-enriched by magnetic-activated cell sorting (MACS) using the W8B2-APC microbead kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the protocol provided by the manufacturer. In brief, at least 10^8 Ficoll-isolated BM cells were incubated with 100 µL of W8B2-APC antibody in phosphate-buffered saline (PBS; Lonza, Verviers, Belgium) together with blocking reagent for 30 min. After washing, the cells were incubated with 200 µL of anti-APC microbeads for 15 min and loaded onto a LS MACS column. $W8B2^+$ cells retained on the column were eluted with 3 mL of PBS containing 0.5 M EDTA and 0.5% bovine serum albumin (BSA; Sigma-Aldrich, Taufkirchen, Germany). In the next step, the cells were stained with the indicated antibody conjugates and used for flow cytometric analysis and fluorescence-activated cell sorting (FACS). For FACSorting, cells were stained with W8B2-APC and CD56-FITC and fractionated into $W8B2^+CD56^-$ and $W8B2^+CD56^+$ populations using a FACSAria cell sorter (Becton Dickinson, Heidelberg, Germany).

CFU-F colonies

Colony forming unit fibroblasts (CFU-F) assays were performed by plating either 1×10^5 unselected or 500–5,000 FACS-selected BM mononuclear cells in T-25 flasks, coated with 0.1% gelatin, containing Knockout™ medium supplemented with 20% knockout serum replacement (Invitrogen, Karlsruhe, Germany), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol (Sigma-Aldrich, Taufkirchen, Germany), 1% nonessential amino acids, and 5 ng/mL human basic fibroblast growth factor (bFGF; CellSystems, St. Katharinen, Germany). After 12–14 days of culture, adherent cells were washed twice with PBS, fixed with methanol (Sigma-Aldrich) for 5 min at room temperature, air-dried, and stained with Giemsa solution (Merck, Darmstadt, Germany). CFU-F colonies were macroscopically enumerated.

Reagents

Antibody conjugates used in these studies include: MSCA-1-APC, MSCA-1-PE, CD271-APC (Miltenyi Biotec Inc., Bergisch Gladbach, Germany), SSEA-3-PE, SSEA-4-PE, TRA-1-60-PE, TRA-1-81-PE, CD56-FITC (Becton Dickinson).

Antigen identification

The 10^8 WERI-RB-1 cells were washed 2 times in PBS. Cells were lysed by incubation in SDS-RIPA lysis buffer (50 mM HEPES, pH = 7.4; 1% desoxycholinacid, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM EGTA, 100 mM NaF with 1% PMSF, and 0.1% aprotinin) for 5 min at room temperature. Protein solutions were centrifuged at 13,000*g* for 45 min at 4°C. The supernatants were stored at −20°C until used for separation on SDS-PAGE. The amount of protein in cell lysate was determined in duplicate in a microplate reader using the Bio-Rad D_c protein assay (assay kit and reader from Bio-Rad, München, Germany). Crude lysates and supernatants from phosphatidylinositol-specific phospholipase C (PI-PLC)-treated WERI-RB-1 cells were separated on 10% SDS-PAGE gels and the resulting bands were visualized by silver staining. The bands at 68 kDa were cut and used for fingerprint analysis. In brief, the bands were excised and washed 3 times in a 60:40 solution of 100 mM ammonium bicarbonate (pH 7.8)/100% acetonitrile for 1 h at room temperature. The solution was removed and the gel pieces were vacuum-dried for 25 min in vacuum concentrator (Eppendorf Hamburg, Germany). The gel bands were then rehydrated and reduced with 100 mM DTT at 56°C and treated with iodoacetamide for 20 min at RT. Digestion was performed with 250 ng trypsin overnight at 37°C. Peptides

were concentrated and spotted on a gold target, mixed with 2,5-dihydroxybenzoic acid (DHB) solution. Peptide mass maps of tryptic peptides were generated by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) using a Bruker Reflex IV mass spectrometer (Bruker Daltonics, Bremen, Germany). The resulting data were used to perform searches of the Swiss Prot and TrEMBL databases using the program MASCOT (www.matrixscience.com). Identification was based on Mascot score, observed p*I* and M_r (kDa), number of matching peptide masses, and total percentage of the amino acid sequence that those peptides covered.

Reverse transcriptase PCR analysis

Total RNA was isolated from the adherent cells by using RNeasy mini kit (Qiagen, Hilden, Germany) and treated with DNase I (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. One microgram of RNA was reverse-transcribed using the ImProm-II Reverse Transcription system (Promega, Mannheim, Germany) as described by the manufacturer's protocol. The PCR was carried out in a 25-µL reaction mixture containing 1 µL cDNA as template, specific oligonucleotide primer pairs (Table 1), and AmpliTaq DNA Polymerase (Applied Biosystems, Darmstadt, Germany) for 35 cycles at 90°C for 1 min, at 55°C or 60°C for 30 s, and at 72°C for 1 min. Ten microliters of the amplification products were separated by electrophoresis in 2% agarose gels and visualized by ethidium bromide staining.

Generation of HEK-293/huTNAP transfectant cell line

Transfection-ready GFP-tagged ORF clone of *Homo sapiens* alkaline phosphatase, tissue non-specific alkaline phosphatase (TNAP, accession number: NM_000478) was obtained from OriGene, Rockville, MD. HEK-293 cells were selected for the transfection because of their lack of reactivity with antibody W8B2. The 10^5 cells were transfected according to the manufacturer's protocol in the presence of transfectant MegaTran 1.0 (OriGene, Rockville, MD) in RPMI 1640 medium containing 10% FBS (PAA Laboratories, Pasching, Austria). The transfected cells and mock-transfected cells were grown for additional 2 days in the same medium before culture of cells in selection medium containing neomycin analog G418. After 3 days of culture, cells were stained with antibody W8B2-APC and analyzed by flow cytometry for GFP and MSCA-1 expression.

Table 1. Reverse Transcriptase Polymerase Chain Reaction Primer Sequences

mRNA Targets	*Oligonucleotides (5′–3′)*	*Product size (bp)*
GAPDH	F: gccaaggtcatccatgacaactttgg R: gcctgcttcaccaccttcttgatgtc	313
TNAP	F: ggacatgcagtacgagctga R: ccaccaaatgtgaagacgtg	282

Primer sequences for TNAP were used as described by Arpornmaeklong et al. [36]. GAPDH primers were used as described by Battula et al. [6].

TNAP, tissue non-specific alkaline phosphatase.

Immunofluorescence staining for flow cytometry

BM cells were washed twice with PBS containing 1% FBS and 0.01% NaN_3 (FACS buffer) and incubated with polyglobin for 15 min on ice to block the nonspecific binding. In the next step, the cells were stained with antibody conjugates W8B2-APC, CD56-FITC, and either SSEA-4 PE, SSEA-3 PE, TRA-1-60-PE, or TRA-1-81-PE for 15 min on ice. After washing, the cells were transferred into Falcon tubes and 1×10^6 cells were analyzed on a FACSCanto flow cytometer (Becton Dickinson, Heidelberg, Germany) using the FCS express software (De Novo Software, Ontario, Canada). For triple staining, BM cells were labeled with W8B2-APC, SSEA-3-PE, and CD56-FITC, gated on the $W8B2^+$ population (R2) and displayed in the plot of SSEA-3 versus CD56.

Treatment of cells with phosphatidylinositol-specific phospholipase C

WERI-RB-1 cells were treated with or without 3 U of PI-PLC from *Bacillus cereus* (Invitrogen, Karlsruhe, Germany) and incubated for 1 h in PBS at 37°C. Cells were then centrifuged at 10,000*g* for 3 min. The supernatant was collected and separated on 10% SDS-PAGE. The resulting bands were visualized by silver staining. In addition, treated and untreated cells were stained with W8B2-APC antibody. After washing, cells were analyzed on a FACSCanto flow cytometer.

Alkaline phosphatase assay

$MSCA\text{-}1^+$ and $MSCA\text{-}1^-$ primary bone marrow subsets were sorted onto chamber glass slides (BD Biosciences, Heidelberg, Germany) and assayed for TNAP activity using StemTAG Alkaline Phosphatase Staining Kit (Cell Biolabs, San Diego, CA). Staining was performed according to the manufacturer's instructions. Cells were photographed with a Zeiss Axiovert 200 microscope (Carl Zeiss, Oberkochen, Germany).

Preparation of endometrial single cell suspension

The endometrium was scraped from the myometrium and dissociated into single cell suspensions using enzymatic and mechanical means as previously described [28] using collagenase 3 (2.5 mg/mL; Worthington Biochemical Corp., Lakewood, NJ), DNase I (1 mg/mL; Worthington Biochemical Corp, New Jersey) in 0.1 M PBS (pH 7.4) for 45–60 min, followed by collagenase 2 (4 mg/mL; Worthington Biochemical Corp.) and DNase I in 0.1 M PBS (pH 7.4) for 20–40 min. Red blood cells and dead cells were removed using Ficoll-Paque density gradient separation (GE Healthcare, Uppsala, Sweden) and leukocytes with CD45 magnetic beads (Dynabead, Invitrogen) as described [29].

Flow cytometric analysis of purified human endometrial cells

Freshly isolated endometrial cell and epithelial cell suspensions (5×10^5 cells/mL) were incubated with antibodies against W8B2, IgG_1 (isotype control; Dako Cytomation, Glostrup, Denmark), or EpCAM (positive control epithelial cell marker, clone BerEP4; Dako) for 45 min at 4°C. Cells were subsequently incubated with PE-conjugated sheep anti-mouse IgG (1 µL; Dako) for 30 min at 4°C and resuspended

in 5% fetal calf serum (Invitrogen)/PBS for flow cytometric analysis using a MoFlo flow cytometer and Summit software (v4.3, Dako Cytomation Inc., Fort Collins, CO).

Immunohistochemistry of endometrial tissue

Frozen sections of human endometrium (5 µm) were fixed in acetone for 5 min at room temperature (RT). Sections were then sequentially incubated with 0.3% hydrogen peroxide (Orion Laboratories, Welshpool, Australia) and protein block (Dako) for 10 min each at RT. W8B2 (undiluted supernatant) and IgG_1 isotype control antibodies were diluted in 0.1% BSA/PBS, and incubated for 1 h at 37°C. An EpCAM reactive antibody (1:20 dilution; Dako) was the positive control. Sections were then incubated with Dako-biotinylated Streptavidin LSAB $^+$ System – HRP Kit for 30 min. Staining was visualized using 3,3′-diaminobenzidine (DAB) tablets and urea peroxidase (both from Sigma, St. Louis, MO) for 5 min at RT. Cells were counterstained with Harris's hematoxylin (Amber Scientific, Midvale, Australia) for 30 s and washed with distilled H_2O. Sections were mounted in DPX (BDH, VWR International Ltd., Poole, UK) and examined using a Zeiss, AxioVision Release 4.6 (Axioskop; Carl Zeiss, Oberkochen, Germany).

Results

MSCA-1 is identical to TNAP

Our preliminary data have shown that W8B2-defined antigen is sensitive to phosphoinositol phospholipase C (PI-PLC) treatment. After the cleavage of glycophosphatidylinositol (GPI)-linked proteins, the staining of cells with W8B2 antibody was completely abrogated (Fig. 1A). Therefore, WERI-RB-1 cells were treated with PI-PLC and the resulting supernatant was separated by SDS-PAGE. The separation revealed a prominent band at molecular mass of about 68 kDa resulting from both samples (Fig. 1B). Fingerprint analysis of the cut bands revealed tryptic peptides from alkaline phosphatase. In addition, peptides from human serum albumin (HSA) could be also identified as a minor fraction (data not shown). To confirm the specificity of antibody W8B2 for TNAP, HEK-293 cells were transfected with a PrecisionShuttle pCMV6-AC-GFP Destination Vector (OriGene, Rockville, MD) containing the complete coding sequence of human TNAP. Three days after transfection, ~30% of transfected HEK-293 cells expressed GFP and reacted with W8B2 antibody (Fig. 1C). Mock-transfected cells showed only baseline reactivity with this antibody (Fig. 1D). These data demonstrate that the W8B2 target MSCA-1 is identical to TNAP. In line with the specific protein expression, reverse transcriptase analysis of TNAP mRNA expression revealed a TNAP-specific band only in HEK-293/huTNAP cells but not in mock-transfected HEK-293 cells (Fig. 1E).

Cell surface TNAP/MSCA-1 in BM cells shows enzymatic activity

We have previously shown that BM-derived MSC can be prospectively isolated using the MSCA-1-reactive antibody W8B2 [11,18]. To study whether alkaline phosphatase expressed on the surface of TNAP/MSCA-1$^+$ cells is functional, BM cells were stained with W8B2-APC and CD56-FITC, fractionated by FACS into TNAP/MSCA-1$^+$ and TNAP/

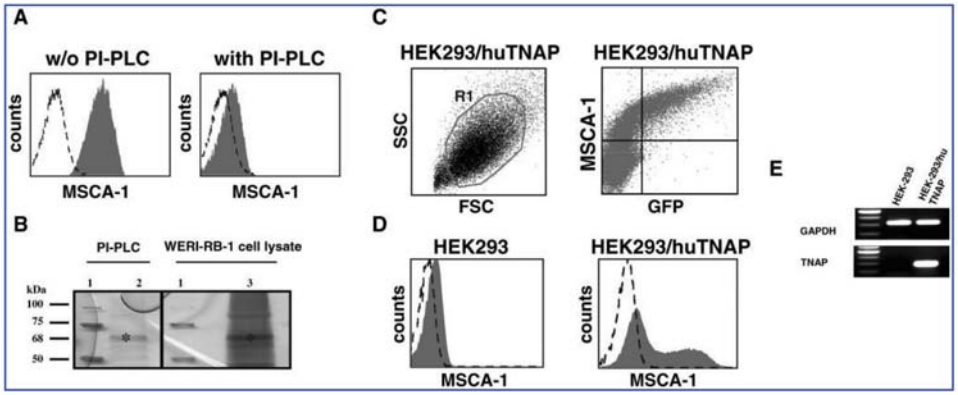

FIG. 1. Mesenchymal stem cell antigen-1 (MSCA-1) is identical to tissue non-specific alkaline phosphatase (TNAP). W8B2-reactive WERI-RB1 cells were used for the identification of the MSCA-1 antigen. (**A**) WERI-RB-1 cells stained with antibody W8B2 before and after treatment with PI-PLC. Open histograms represent control staining. (**B**) Supernatant from PI-PLC-treated WERI-RB-1 cells (lane 2) and WERI-RB-1 whole cell lysates (lane 3) were separated on 10% SDS gel and visualized by silver staining. Molecular mass markers are shown on the left (lane 1). * indicates position of the 68-kDa band used for matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis. (**C**) Display of SSC versus FSC of HEK-293 cells transfected with TNAP. Cells in gate R1 are shown as plots of GFP versus TNAP/MSCA-1. Approximately 30% of transfected cells were GFP$^+$W8B2$^+$. (**D**) Expression of MSCA-1 on HEK-293/huTNAP cells (right); lack of expression of TNAP/MSCA-1 on mock-transfected HEK-293 cells (left). (**E**) RT-PCR analysis of TNAP mRNA expression. TNAP expression was detected only in the transfectant cell line (HEK-293/huTNAP). Expression of GAPDH served as a positive control.

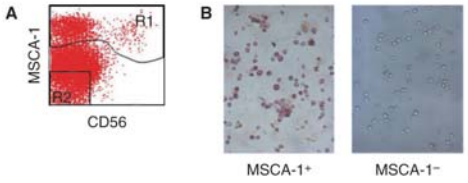

FIG. 2. Enzymatic activity of cell surface tissue non-specific alkaline phosphatase (TNAP)/mesenchymal stem cell antigen-1 (MSCA-1) in bone marrow (BM) cells. BM cells were stained with W8B2-APC and fractionated by FACS into TNAP/MSCA-1$^+$ (R1) and TNAP/MSCA-1$^-$ (R2) populations. (**A**) Display of TNAP/MSCA-1 versus CD56 on MACS-selected BM cells. (**B**) TNAP/MSCA-1$^+$ and TNAP/MSCA-1$^-$ cell subsets were sorted onto chamber slides and stained for membrane TNAP activity. Note the exclusive presence of enzymatically active TNAP in the TNAP/MSCA-1$^+$ subset. Magnification: 200×.

MSCA-1$^-$ cells, and analyzed for cell surface TNAP activity (Fig. 2A). As shown in Figure 2B, specific TNAP activity was detected only on the surface of MSCA-1$^+$ but not on TNAP/MSCA-1$^-$ BM cells. This demonstrates that antibody W8B2 detects functional TNAP on BM cells.

TNAP/MSCA-1$^+$ BM cells express SSEA-3 but not SSEA-4, TRA-1-60, or TRA-1-81

Next, we tested the coexpression of the ES cell markers SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 on TNAP$^+$ BM cells. As shown in Figure 3, a prominent coexpression of SSEA-3 on the majority of TNAP$^+$ BM cells is detected (79% of TNAP$^+$ cells). However, a minority (21% of TNAP$^+$ cells) were negative for SSEA-3, indicating that SSEA-3 is a novel marker for MSC subsets. In contrast to SSEA-3, the other embryonic markers were negative on TNAP$^+$ cells. To determine whether TNAP/MSCA-1$^+$CD56$^+$ MSC coexpress SSEA-3, BM cells were triple-stained with W8B2-APC, SSEA-3-PE, and CD56-FITC and gated on the TNAP$^+$ population. As shown in the plot of SSEA-3 versus CD56 (Fig. 3B), cells of the rare CD56$^+$ MSC subset do not coexpress SSEA-3.

TNAP/MSCA-1 is differentially expressed on BM cells from different sources

We have recently shown that CD271^{bright}MSCA-1^{dim}CD56$^+$ cells of primary femur-derived BM gave rise to about 2-fold higher CFU-F compared to CD271^{bright}MSCA-1^{bright}CD56$^-$ cells [18]. Here, we analyzed the spatial distribution and clonogenic potential of these subsets in different localizations of human bone including acetabulum, trochanter, and trabeculum, and femur. Staining of BM cells with antibodies against CD271, MSCA-1, and CD56 revealed the presence of TNAP/MSCA-1$^+$CD56$^+$ and TNAP/MSCA-1$^+$CD56$^-$ subsets, a finding which is in agreement with our previous studies on femur-derived BM cells (Fig. 4A and 4B). Trochanter and trabeculum contained the highest percentage of TNAP/MSCA-1$^+$CD56$^-$ cells (71% and 64%, respectively), whereas TNAP/MSCA-1$^+$CD56$^+$ cells

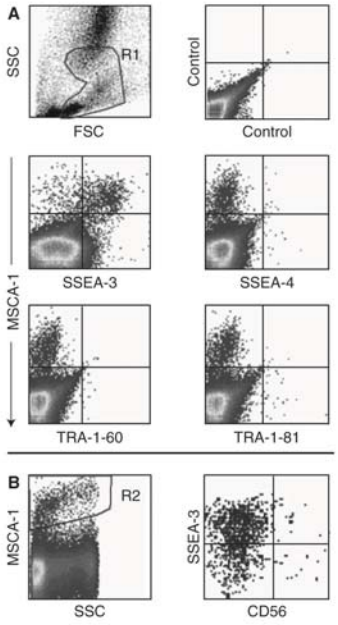

FIG. 3. Tissue non-specific alkaline phosphatase (TNAP)/mesenchymal stem cell antigen-1 (MSCA-1)$^+$ bone marrow (BM) cells express SSEA-3 but not SSEA-4, TRA-1-60, or TRA-1-81. (**A**) BM cells stained with W8B2-APC and PE-conjugated antibodies against SSEA-3, SSEA-4, TRA-1-60, and TRA-1-80 were gated in the dual scatter plot (R1) and analyzed for coexpression of TNAP/MSCA-1 and the indicated markers. Control staining is shown in the upper right plot. (**B**) BM cells were triple-stained with W8B2-APC, SSEA-3-PE, and CD56-FITC, gated on the W8B2$^+$ population (R2) and displayed in the plot of SSEA-3 versus CD56. Note that CD56$^+$ MSC lack coexpression of SSEA-3.

were enriched in femur (58%). The level of TNAP expression in the CD56$^+$ population varied considerably: CD56$^+$ cells of acetabulum expressed high levels of TNAP/MSCA-1, whereas trochanter, femur, and trabeculum CD56$^+$ cells expressed this ectoenzyme only at low levels (Fig. 4B). CFU-F assays of 2 separate experiments revealed that acetabulum CD56$^+$ cells did not only express the highest TNAP levels but also give rise to the highest frequency of colonies. In contrast, femur-derived CD56$^+$ cells expressed the lowest TNAP levels and showed the lowest cloning efficiency (Fig. 4C). In agreement with previous results, CD56$^+$ cells of the studied 4 bone sources in all cases gave rise to about twice as many CFU-F as CD56$^-$ cells.

TNAP/MSCA-1 is expressed on endometrial MSC-like cells and endometrial epithelial cells

Flow cytometric analysis revealed that antibody W8B2 detected a small population of freshly isolated human

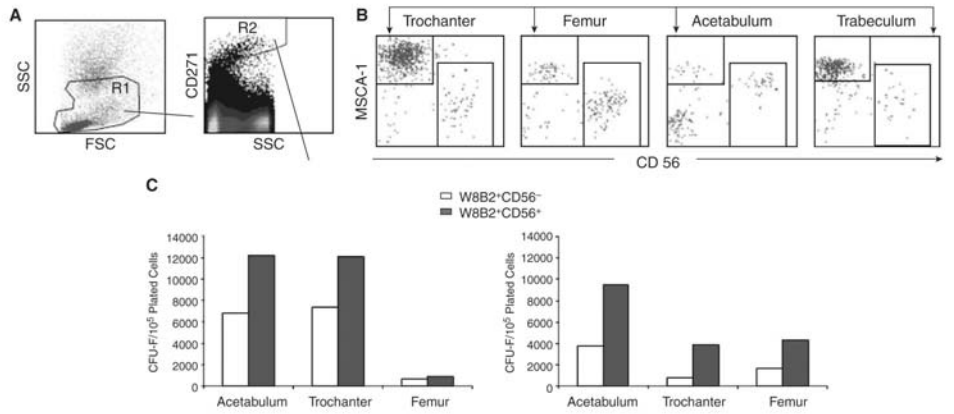

FIG. 4. Tissue non-specific alkaline phosphatase (TNAP) is differentially expressed on bone marrow (BM) cells from different sources. Dual scatter gated BM cells (R1) of femur, acetabulum, trabeculum, and trochanter stained with antibodies against CD271-APC, CD56-FITC, and TNAP/mesenchymal stem cell antigen-1 (MSCA-1) were gated on the CD271^{bright} (R2) subset and analyzed for coexpression of CD56 and MSCA-1. (**A**) Display of BM cells in the dual scatter (left) plot and the plot of SSC versus CD271^{bright} (right). (**B**) Coexpression of CD56 and MSCA-1 on CD271^{bright} cells of different BM sources. (**C**) Colony forming unit fibroblasts (CFU-F; normalized to 1×10^5 plated cells) of FACS-sorted TNAP/MSCA-1$^+$CD56$^-$ (white bars) and TNAP/MSCA-1$^+$CD56$^+$ (gray bars) cells derived from femur, acetabulum, and trochanter. CFU-F numbers were determined as described in the Materials and Methods. The plots represent the results from 2 separate experiments.

endometrial cells (Fig. 5A). To investigate whether TNAP is expressed on CD146$^+$ endometrial MSC-like cells [29], cells were double-stained with TNAP-(W8B2) and CD146-reactive antibodies and analyzed by flow cytometry. Figure 5B shows that a small CD146$^+$ population (2.5%) coexpressed TNAP, a similar proportion as documented for CD146$^+$PDGFRβ$^+$ endometrial MSC-like cells [29]. Immunohistochemistry staining showed that TNAP is expressed at high levels on the luminal surface of the glandular epithelium in addition to a weaker expression in blood vessels (Fig. 5C). In agreement with the previously described colocalization of CD146 and PDGFRβ on endometrial MSC-like cells, W8B2 antibody showed also some immunoreactivity adjacent to the vascular lumen composed of pericytes and/or perivascular cells (Fig. 5D).

Discussion

In the present study, we have shown that MSCA-1 is identical to the TNAP. This antigen is a GPI-anchored membrane protein that can be cleaved by treatment with PI-PLC [21,22]. In agreement with this feature, MALDI-TOF MS analysis revealed TNAP sequences in the supernatant of WERI-RB-1 cells treated with PI-PLC. To confirm the identity of TNAP and MSCA-1, HEK-293 cells were transfected with a plasmid containing the complete coding sequence of human TNAP. Flow cytometry analysis showed that MSCA-1-specific antibody W8B2 selectively recognized HEK-293/huTNAP transfectant cells but not the parent cells. In agreement with the TNAP specificity, we could demonstrate that only W8B2$^+$ but not W8B2$^-$ bone marrow (BM) cells express the enzymatically active form of the TNAP. In addition, we have shown in different bone sources that only W8B2$^+$ cells gave rise to MSC. In conclusion, we have proved that antibody W8B2 detects functional TNAP on the surface of BM-derived MSC.

TNAP catalyzes the hydrolysis of phosphomonoesters with release of Pi [19,20] and is known to play an important role in bone remodeling by regulating the extracellular concentrations of inorganic pyrophosphate (PPi). PPi suppresses the formation and growth of hydroxyapatite crystals needed for osteoblasts to mineralize the bone matrix [30–33]. Recently, it was shown that TNAP and low-affinity nerve growth factor receptor (LNGFR; CD271) are up-regulated in mineralizing cells derived from jaw periosteum-derived cells (JPCs). This suggests that TNAP/MSCA-1 and LNGFR are involved in calcification and may serve as a differentiation marker of the mineralizing JPCs [34].

The biological role of TNAP in MSC remains to be clarified. The analysis of the spatial distribution in vivo and determination of in vitro parameters may facilitate to elucidate the physiological function of the TNAP. We observed a negative correlation between the intensity of TNAP expression in the different anatomical sites of the hip joint and the CFU-F frequency. Thus, in acetabulum the level of TNAP expression was lowest whereas the frequency of CFU-F was highest. This is in accordance with the model favored by Gronthos et al. [35] who proposed that early MSC progenitor cells lack TNAP expression but acquire this enzyme during osteogenic differentiation. Our data support the view that the expression of TNAP is restricted to MSC of a later differentiation status. In this respect, it is of interest that the recently identified CD56$^+$ and CD56$^-$ MSC subsets show differential expression of TNAP [18]. Thus, the CD56$^-$ population with adipocyte differentiation capacity expressed high levels of TNAP whereas the

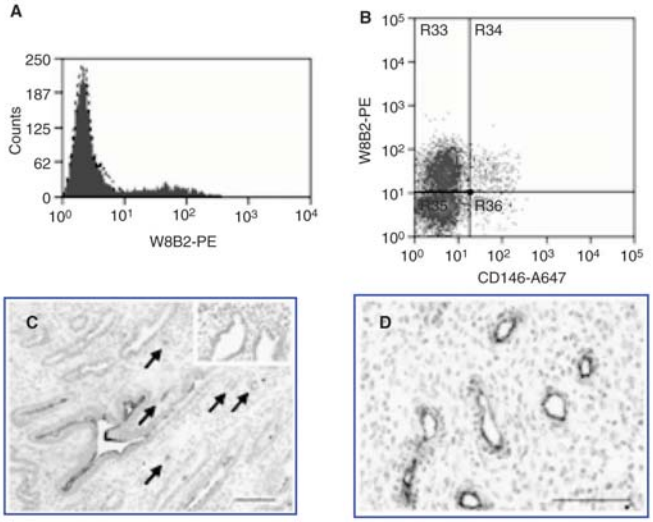

FIG. 5. Tissue non-specific alkaline phosphatase (TNAP)/mesenchymal stem cell antigen-1 (MSCA-1) is expressed on endometrial epithelium and mesenchymal stem/stromal cells (MSC)-like cells. (**A**) Flow cytometric analysis of unfractionated endometrial cells stained with antibody W8B2. (**B**) Dual color plot of $EpCAM^-$ endometrial stromal cells stained with W8B2 and CD146, showing a small $W8B2^+CD146^+$ cell population. (**C**) Immunoreactivity of antibody W8B2 with the luminal surface of glandular epithelium and localization in all vessels (arrows) during the mid-proliferative phase of the menstrual cycle. Inset: negative control IgG_1. Scale: 100 μm. (**D**) Perivascular immunostaining of a spiral arteriole showing endothelial/pericyte immunoreactivity. Scale: 100 μm.

$CD56^+$ subset expressing low levels of this ectoenzyme contained progenitors committed to the chondrocyte lineage. Osteogenic progenitors were found in both fractions. Whether the lower level of TNAP expression in the $CD56^+$ subset is correlated with an earlier maturation status remains to be determined.

In this report, we describe the differential expression of TNAP on $CD56^+$ and $CD56^-$ MSC derived from different sites of bone including acetabulum, trochanter, trabeculum, and femur. In the $CD56^+$ subsets, the strongest expression of TNAP was detected in acetabulum, whereas in the $CD56^-$ subsets, the highest levels of TNAP expression were recorded in trochanter. Interestingly, the CFU-F were more enriched in acetabulum, independent of the analyzed fraction. Although it is likely that osteoprogenitor cells are enriched in acetabulum because this site contains less marrow and more bone than the other studied sites, specific differentiation assays are necessary to confirm this speculation.

Arpornmaeklong et al. described that a subpopulation of human ES cells induced to differentiate into MSC expresses alkaline phosphatase upon osteogenic lineage commitment. As a consequence, they used TNAP as a key marker for the isolation of ES cell-derived osteoprogenitor cells [36]. Jones et al. used the fibroblast marker D7-FIB as a surface antigen for MSC isolation and reported that BM-derived MSC are positive for alkaline phosphatase enzyme activity [37]. However, they did not show that TNAP is a selection marker for MSC isolation and did not demonstrate TNAP expression on the cell surface. Our report is therefore the first that describes successful isolation of MSC from primary bone marrow tissue.

As the TNAP is expressed at high levels on the surface of ES cells [27], it was of interest to investigate the coexpression of other ES cell markers on $TNAP^+$ BM cells including SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81. Of these, TRA-1-60, TRA-1-81, and SSEA-4 were negative on the $TNAP^+$ BM cells. The fact that SSEA-4 was not expressed on primary BM-derived MSC is in contradiction to the data of Gang et al. who reported SSEA-4 expression on these cells and introduced this molecule as a suitable marker for the prospective isolation of MSC [13]. Most likely, these authors used an antibody from another source. In fact, screening of BM cells with a different antibody claimed to be specific for SSEA-4, stained ~20% of BM cells. The antibody used in our studies was active since it strongly reacted with ES cells (unpublished). It is therefore likely that SSEA-4 is not expressed on BM-derived MSC. In contrast to the other ES cell-specific markers, SSEA-3 was found to be expressed on the majority of $TNAP^+$ cells (~80%). Interestingly, the $TNAP^+CD56^+$ MSC did not express SSEA-3. Thus, SSEA-3 is a marker that defines a novel subset of BM-derived MSC. Current studies are ongoing to determine the clonogenic and differentiation potential of $TNAP^+SSEA\text{-}3^+$ and $TNAP^+SSEA\text{-}3^-$ fractions.

In the past, endometrial decidua was reported to express TNAP [25]. Here, we analyzed in more detail the TNAP staining pattern of endometrial tissue and found specific and heterogeneous expression on the luminal surface of glandular epithelia. TNAP is therefore a suitable marker for the isolation of a subset of endometrial glandular epithelial cells. More importantly, TNAP is also expressed on endometrial perivascular cells, where interestingly endometrial MSC-like cells are located [29]. Furthermore, the proportion of $W8B2^+CD146^+$ endometrial stromal cells is very similar to the proportion of $CD146^+PDGFR\beta^+$ MSC-like cells found in human endometrium, suggesting that endometrial MSC-like cells express TNAP and that combined with CD146 this ectoenzyme may be a suitable marker for the isolation from the $EpCAM^-$ endometrial stromal cell fraction. The exclusive expression of TNAP in the $CD146^+$ subset, but not on other MSC-like/fibroblast-like cells, suggests

that TNAP is developmentally expressed on MSC/pericyte progenitor cells, which is down-regulated during further differentiation.

The precise role of the CD146$^+$PDGFR-β^+ subset of endometrium remains to be demonstrated. One possibility is that they are derived from remnant fetal stem cells, which persist after uterine development [38]. These cells have multilineage potential [29] and may originate from the blood vessel wall [39]. Another possibility is that these cells are derived from bone marrow MSC migrating into endometrial tissue during every menstrual cycle [40,41]. The fact that TNAP is expressed in this cell subset suggests that this ectoenzyme plays an important role in the differentiation and endometrial regeneration.

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Address correspondence to:
Dr. Hans-Jörg Bühring
Department of Internal Medicine II
Division of Hematology, Oncology, Immunology, and Rheumatology
Laboratory for Stem Cell Research
University Clinic of Tübingen
Otfried-Müller-Str. 10
Tübingen 72076
Germany

E-mail: hans-joerg.buehring@uni-tuebingen.de

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