Candidate Markers of Epithelial Stem/progenitor Cells in Human Endometrium and Endometrial Cancer

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Table of contents

Abstract

The human endometrium is the highly regenerative mucosal lining of the uterus which undergoes cyclical regeneration following menstruation and parturition. Atrophic endometrium also regenerates when post-menopausal women take estrogen therapy. A rare population of endometrial epithelial stem/progenitor cells are likely involved in this remarkable regeneration. However, their precise origin and location are unknown. Endometrial cancer is the most common gynaecological malignancy in women. Genetic alterations involving the stem cell compartment are thought responsible for this cancer.

At the beginning of this study, there were no known markers for the prospective isolation of human endometrial epithelial stem/progenitor cells. The overall aim of this thesis therefore, was to identify surface markers to purify endometrial epithelial stem/progenitor cells and endometrial cancer stem cells. It was hypothesised that the epithelium in the basalis of pre-menopausal endometrium and post-menopausal endometrium are similar and contain stem/progenitor cells with common gene expression profiles.

To enable the identification of endometrial epithelial stem/progenitor cells, a robust isolation and characterisation techniques using enzyme digestion, differential filtration and flow cytometry sorting was developed. A transcriptional study was then undertaken which identified differential expression of 22 Wnt signalling pathway genes between epithelial cells isolated from pre- and post-menopausal endometrium. One gene, Axin2, a negative regulator of the Wnt pathway was specifically localised to the nucleus of epithelial cells in the basalis of pre-menopausal endometrium and post-menopausal endometrium, possibly to regulate cytoplasmic β-catenin and stabilise Wnt downstream target genes.

The surface marker, N-cadherin selected from the gene list, enriched for a small population of endometrial epithelial cells with stem cell activity. N-cadherin was localised to epithelial glands deep in the basalis layer adjacent to the myometrium, with a gradient of reducing expression through to the functionalis and luminal epithelium. A common gene pathway was identified in the epithelium of the basalis of pre-menopausal endometrium and in post-menopausal endometrium, confirming the likely basalis location of resident epithelial stem/progenitor cells.

This thesis also investigated known cancer stem cell markers (CD24, CD49f, CD29, CD44) for their potential to isolate cancer stem cells from primary endometrial cancer and endometrial cancer cell lines. The levels of surface marker expression greatly varied between primary endometrial cancer cells and the cell lines. Sorted endometrial cancer cell lines exhibited colony-forming activity and sphere formation more readily compared to primary endometrial cancer cells. However, the markers examined did not enrich for cancer stem cell activity. Stromal feeder layers were necessary to support clonal cultures of sorted primary endometrial cancer cells, however further work is required to identify potential endometrial cancer stem cell markers. Future studies should also examine N-cadherin for its ability to isolate endometrial cancer stem cell population.

This investigation supports the concept that endometrial epithelial stem/progenitor cells exist in pre- and post-menopausal endometrium which can be partially purified using a single marker N-cadherin. Identification of this marker lays the foundation for further characterisation of the molecular and cellular phenotype of human endometrial epithelial stem/progenitor cells and examines their role in endometrial regeneration and in gynaecological disorders such as endometrial cancer.

General Declaration

Monash University
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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 one original paper being reviewed by a peer reviewed journal and three unpublished manuscripts in preparation for eventual publication. The core theme of the thesis is epithelial stem/progenitor cells in human endometrium and endometrial cancer. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Ritchie Centre, Monash Institute of Medical Research under the main supervisor Dr Caroline Gargett.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapter 2, 3 and 4, my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status	Nature and extent of candidate's contribution
2	Isolation, culture and characterisation of human endometrial epithelial cells.	In preparation	Responsible for refining the research question, designing and performing the experiments, collecting data for all figures except Supplemental figure 2, interpreting and preparing all figures and tables and manuscript writing (75% contribution)
3	Differential expression of Wnt signalling molecules between pre- and post-menopausal endometrial epithelial cells suggests a population of putative epithelial stem/progenitor cells reside in the basalis layer	Under revision with Endocrinology	Responsible for refining the research question, performing the experiments, collecting and analysing and generating data for all figures except Figure 1, and manuscript writing (80% contribution)
4	N-cadherin isolates putative human endometrial epithelial stem/progenitor cells.	In preparation	Responsible for refining the research question, performing the experiments, collecting and analysing and generating data for all figures except Figure 1, and manuscript writing (80% contribution)

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

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Structure of Thesis

In compliance with Monash University Doctorate regulations, this thesis consists of unpublished work relating to "Candidate markers of epithelial stem/progenitor cells in human endometrium and endometrial cancer".

Chapter 1 - General introduction. *Written as a chapter*

Chapter 2 - Isolation, culture and characterisation of human endometrial epithelial cells. *Written as a manuscript in preparation for publication*

Chapter 3 - Differential expression of Wnt signalling molecules between pre- and post-menopausal endometrial epithelial cells suggests a population of putative epithelial stem/progenitor cells reside in the basalis layer. *Written as a manuscript, re-submitting to Endocrinology*

Chapter 4 - N-cadherin isolates putative human endometrial epithelial stem/progenitor cells. *Written as a manuscript in preparation for publication*

Chapter 5 - Candidate markers of human endometrial cancer stem/progenitor cells. *Written as a manuscript in preparation for publication*

Chapter 6 - General discussion. *Written as a chapter*

List of publications

Peer reviewed publication

Differential expression of Wnt signalling molecules between pre- and post-menopausal endometrial epithelial cells suggests a population of putative epithelial stem/progenitor cells reside in the basalis layer. *Endocrinology* (under review).

Conference Abstracts (presenting author first)

Nguyen, H.P.T., Sprung, C.N., Watkins, N.D. and Gargett, C.E. Gene expression profiling to identify human endometrial epithelial stem/progenitor cells. Southern Health Research Week, Southern Health, Monash Medical Centre. 9th – 13th May 2011. Melbourne, Australia.

Nguyen, H.P.T., Sprung, C.N., Watkins, N.D. and Gargett, C.E. Gene expression profiling to identify human endometrial epithelial stem/progenitor cells. Society for Gynecologic Investigation 2011 Annual Meeting. 16th – 19th March 2011, Miami, Florida, USA.

Nguyen, H.P.T. and Gargett, C.E. A model to study endometrial cancer stem-like cells. ASSCR Second Annual Meeting. 22nd – 24th November 2009, Canberra, Australia.

Nguyen, H.P.T. and Gargett, C.E. Approaches to identify epithelial stem/progenitor cells in the normal and malignant endometrium. Southern Health Research Week, Southern Health, Monash Medical Centre. 4th – 8th May 2009, Melbourne, Australia. 1st Prize.

Nguyen, H.P.T. and Gargett, C.E. Approaches to identify epithelial stem/progenitor cells in the normal and malignant endometrium. The 4th Australian Health and Medical Research Congress. 16th – 21st November 2008, Brisbane, Australia.

Other peer reviewed publications during PhD

Gargett, C.E., Schwab, K.E., Zillwood, R.M, **Nguyen, H.P.T.** and Wu, D. Isolation and culture of epithelial progenitors and mesenchymal stem cells from human endometrium. *Biology of Reproduction*, 2009: 80(6) p1136-45. (Appendix 1).

Other abstracts during PhD

Nguyen, H.P.T. and Gargett, C.E. Identifying epithelial stem/progenitor cells in the normal and malignant endometrium. *10th World Congress of Endometriosis.* 11th – 14th March 2008, Melbourne, Australia.

Shapira, D.R., **Nguyen, H.P.T.**, Brown, G., Ho, T. and Molloy, P.L. Stem Cell Like Properties Of Some Colorectal Cancer Cell Lines. *31st Australasian Flow Cytometry Group Annual Scientific Meeting.* 13th – 15th August 2008, Fremantle, Australia.

Shapira, D.R., **Nguyen, H.P.T.** and Molloy, P.L. Stem cell like properties of some colorectal cancer cell lines: implications for the role of cancer stem cells in tumour development. *ISAC XXIV International Congres.* 17th – 21st May 2008, Budapest, Hungary.

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To my dearest parents, my late Uncle Tong and grandparents, I would like to dedicate this thesis to you. Thank you for your love and encouragement, for believing in me and to provide me all the opportunities to pursue my dreams.

Abbreviations

2D	Two dimensional
3D	Three dimensional
7AAD	7-Aminoactinomycin D
µg	microgram
µm	micrometre
°C	Degree Celsius
Ab	Antibody
ABCG2	ATP-binding cassette sub-family G, member 2
ANOVA	Analysis of variance
ASC	Adult stem cells
A488	Alexa fluor 488
A647	Alexa fluor 647
APC	*Adenomatous Polyposis Coli* (gene)
APC	Allophycocyanin (fluorochrome)
APC/A750	Allophycocyanin and Alexa Fluor 750
bFGF	Basic fibroblast growth factor
BMI-1	B lymphoma Mo-MLV insertion region 1 homolog
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
BrdU	Bromodeoxyuridine

cDNA	Complementary deoxyribonucleic acid
CD	Cluster differentiation
CE	Colony-forming efficiency
CFU	Colony-forming unit/activity
CK1	Casein kinase-1
CK	Cytokeratin
cm	Centimetre
c-myc	v-myc myelocytomatosis viral oncogene homolog
CO_2	Carbon dioxide
CSC	Cancer stem cells
Ct	Cycle threshold
DAB	3,3'-diaminobenzidine
DAPI	4,6-diamidino-2-phenylindole
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DMEM/F12	Dulbecco's modified eagle's medium F12
DMSO	Dimethyl sulphoxide
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EDTA	Ethylene diamine tetraacetic acid

EnCa	Endometrial cancer
EpCAM	Epithelial cell adhesion molecule
ER	Estrogen receptor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ES	Early-secretory
ESP	Epithelial stem/progenitor cells
FACS	Fluorescence activating cell sorting
FCS	Fetal calf serum
FIGO	The International Federation of Gynaecology and Obstetrics
FGF	Fibroblast growth factor
FSC	Forward scatter
g	Gravitational force
GSK-3β	Glycogen synthase kinase- 3 beta
H_2O_2	Hydrogen peroxide
H&E	Haematoxylin and Eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HER2	Human epidermal growth factor type II receptor
HLA	Human leukocyte antigen
hr	Hour
HRP	Horseradish peroxidase-conjugated streptavidin
HSC	Haematopoietic stem cells

IgG	Immunoglobulin G
IHC	Immunohistochemistry
IF	Immunofluorescence
kDa	Kilo Dalton
LEF	Lymphoid enhancer factor
Lgr5	Leucine-rich repeat containing G protein-coupled receptor 5
LSAB	Labelled streptavidin-biotin
LRC	Label-retaining cells
LRP5/6	Low density lipoprotein (LDL) receptor-related protein (LRP) 5/6
LS	Late-secretory
M	Molar
MACS	Magnetic activating cell sorting
MPC	Magnetic particle collector
mg	Milligram
ml	Millilitre
min	Minute
mM	Millimolar
MMMT	Malignant Mullerian mixed tumour
mRNA	Messenger ribonucleic acid
MS	Mid-secretory
MS sorting	Magnetic bead sorting
MSI	Microsatellite instability

n	Number of samples
NCS	New calf serum
nm	nanometre
NOD-SCID	Non-obese diabetic-severe combined immunodeficient
NOG	NOD/SCID/γ
p	Probability
P	Proliferative
PBS	Phosphate buffered saline
PBA	Protein blocking agent
PD	Population doublings
PDGF	Platelet-derived growth factor
PDGF-Rβ	Platelet-derived growth factor receptor beta
PE	Phycoerythrin
PeCy5.5	Phycoerythrin and Cyanine 5.5
PI	Propidium iodide
PR	Progesterone receptor
PR-A	Progestrone receptor, type A
PR-B	Progesterone receptor, type B
PR-C	Progesterone receptor, type C
Pre-M	Pre-menopausal
Post-M	Post-menopausal
PTEN	Phosphatase and tensin homolog

qRT-PCR	Quantitative reverse transcription-polymerase chain reaction
RMA	Robust multichip average
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Room temperature
RT controls	Reverse transcription controls
RT-PCR	Reverse transcription-polymerase chain reaction
SD	Standard deviation
sec	Second
SEM	Standard error of the mean
SP	Side populations
SPC	Stem/progenitor cells
SSC	Side scatter
TA cells	Transit-amplifying cells
TCF	T cell factor
TCF/LEF	T cell factor/lymphoid enhancer factor
TGF- β	Transforming growth factor-β

Chapter 1

General introduction

1.1 Stem cells

The concept of a stem cell was proposed more than 30 years ago to describe a sub-population of cells that are responsible for cellular turnover (Lajtha, 1979). Since then, stem cells have been defined by their properties of self-renewal, differentiation and high proliferative potential. There are two main types of stem cells, embryonic stem (ES) cells and adult stem cells. Human embryonic stem cells are harvested from the embryo inner cell mass. They are pluripotent, having the potential to produce all cell types of the three germ layers; ectoderm, mesoderm and endoderm (Bongso & Richards, 2004; Trounson, 2006; Slack, 2008). ES cells were first identified in mice (Evans & Kaufman, 1981; Martin, 1981) which soon lead to their discovery in humans (Thomso*n et al.*, 1998; Per*a et al.*, 2000; Trounson, 2006).

In contrast, adult stem cells are multipotent, rare, quiescent, undifferentiated cells residing in many adult tissues and organs (Morrison *et al.*, 1997; Fuchs & Segre, 2000; Eckfeldt *et al.*, 2005). The main function of adult stem cells is to regulate tissue homeostasis and provide replacement cells during cellular turnover, or following acute injury (Morrison *et al.*, 1997; Fuchs & Segre, 2000; Li & Xie, 2005). This thesis will focus on adult stem cells thus the term stem cells will refer to adult stem cells unless stated otherwise.

Stem cells have been identified and characterised in several body organs and tissue-specific systems including the haematopoietic system (Weissman, 2000; Morrison, 2001), breast (Stingl *et al.*, 2001; Shackleton *et al.*, 2006; Stingl *et al.*, 2006), brain, skin, intestine, colon, ovaries and uterus (Fuchs & Segre, 2000; Weissman, 2000; Morrison, 2001; Stingl *et al.*, 2001; Cai *et al.*, 2004; Chan *et al.*, 2004; Shackleton *et al.*, 2006).

Stem cells have the same traits including rare, quiescent and have the ability to maintain the balance of cellular proliferation and differentiation regardless of the body organs or

tissues in which they reside. The defining features of stem cells are the ability to self-renew for the lifetime of the individual and to produce more differentiated daughter cells. Transit-amplifying (TA) cells are committed to differentiate into mature cell types of a tissue in which stem cells reside (Weissman, 2000; Morrison, 2001) (Fig. 1.1).

1.1.1 Epithelial stem cells

Epithelia comprise tightly linked sheets of cells making up the surface and the linings of the body. Epithelia can be multi-stratified or a single simple layer and derived from any of the three germ layers ectoderm, mesoderm and endoderm. They share similar cellular and molecular characteristics (Blanpain *et al.*, 2007). Epithelial cells adhere to a basement membrane where they are supported by nutrients, extracellular matrix and growth factors. Epithelial cells express trans-membrane integrins anchoring them to the basement membrane as well as intercellular adheren junctions which distinguish the apical, basal and lateral surfaces of the cell and are essential for maintaining epithelial cell polarity (Shin *et al.*, 2006; Blanpain *et al.*, 2007). The life span of epithelial cells is generally less than one week, although it varies between different tissue types. Nevertheless, constant cellular replacement of the epithelia is essential to maintain tissue homeostasis (Reya & Clevers, 2005; Blanpain *et al.*, 2007). This continuous cellular replacement suggests the presence of stem cells which have the ability to self-renew, proliferate to produce progenitor cells and differentiate into the mature epithelial cell types of the tissue they reside.

1.1.2 Properties of stem cells

1.1.2.1 Colony-forming unit activity

Colony-forming unit (CFU) activity is defined as the ability of a single cell to initiate a clone of cells when seeded at extremely low density and is a classic *in vitro* stem cell

property. This approach allows separation of clonogenic cells from mature differentiated cells which do not clone (Loeffler & Potten, 1997; Schwab, 2008). CFU have been identified in prostate and ocular epithelium (Pellegrini *et al.*, 1999; Hudson *et al.*, 2000). Colony size can be used to determine the stem cell capacity of the initiating cell. Generally, large dense colonies are initiated by the stem cells whereas more mature progeny (TA cells) initiate small and loosely packed colonies (Hudson *et al.*, 2000; Chan *et al.*, 2004; Schwab, 2008). While CFU activity is a quick and simple measurement, it is not sufficient to distinguish between stem cells and TA cells, thus other stem cell properties should also be assessed (Harrison, 1980; Gargett, 2007).

1.1.2.2 Self-renewal

Self-renewal is an essential hallmark property of stem cells. It is defined as the ability of a cell to undergo asymmetric division to produce one identical stem cell to maintain the stem cell pool and one more mature progenitor daughter cell (Fig. 1.1) (Morrison *et al.*, 1997; Fuchs & Segre, 2000; Reya *et al.*, 2001). In contrast, the transit-amplifying (TA) cells cannot self-renew, have limited proliferative potential, yet can undergo rapid proliferation and differentiate into mature cell types of the tissue in which they reside (Potten & Loeffler, 1990; Jones & Watt, 1993; Gargett, 2007) (Fig. 1.1).

Serial cloning *in vitro* and serial transplantation *in vivo* are the two main criteria use to assess stem cell self-renewal, and the later is considered the gold standard. For example, limiting dilution analyses of a single mammary cell was shown to self-renew at least 10 times *in vitro* with increasing CFU activity with increasing passage (Stingl *et al.*, 2006). Similarly, this serial clonal outgrowth of the same population is observed *in vivo* that a single cell can reconstitute an entire mammary gland which can be serially passaged at the clonal level several times (Shackleton *et al.*, 2006; Stingl *et al.*, 2006). Self-renewal is also

best described in the haematopoietic system where multipotent stem cells can function for at least 15 months after *in vivo* bone marrow reconstitution (Keller & Snodgrass, 1990).

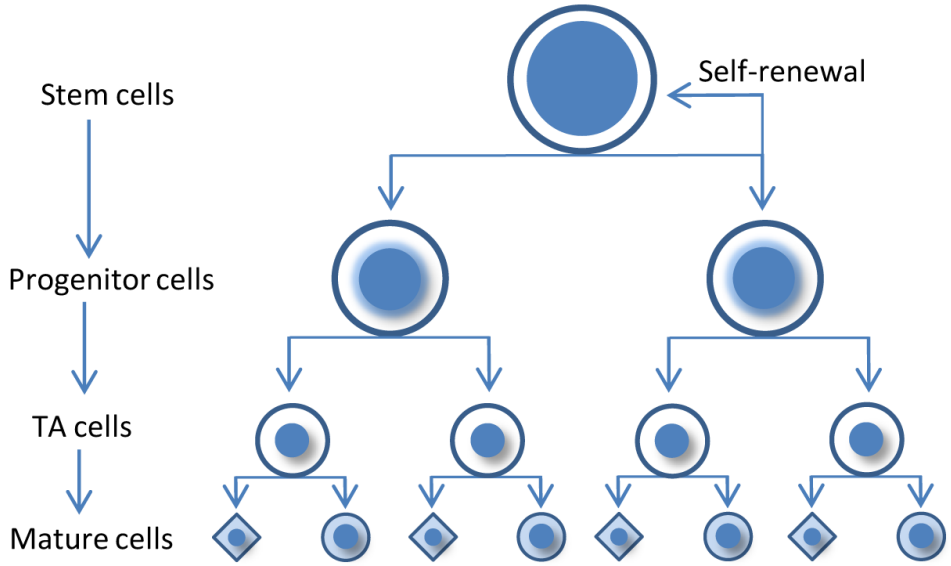


Figure 1.1 Adult stem cell hierarchy. Stem cells have the ability to undergo asymmetric division to self-renew and maintain the stem cell pool. In symmetric division, stem cells proliferate and give rise to progenitor cells which then produces committed transit-amplifying (TA) cells. TA cells undergo rapid proliferation to produce mature cells of the organ/tissue.

1.1.2.3 Proliferative potential

Proliferative potential of a cell is defined as the number of population doublings it produces before reaching senescence. It reflects the number of cell divisions the cell is capable of before reaching terminal differentiation (Potten & Loeffler, 1990). It has been shown that stem cells have the highest proliferative potential which is greater than that of TA cells (Pellegrini *et al.*, 1999). In a study calculating the number of cell divisions occuring in human ocular keratinocytes, it was found that the stem cells could undergo 45 – 50 population doublings compared to their differentiated daughter cells (Pellegrini *et al.*, 1999). Similarly, a thousand human hair follicles could be generated from the progeny of a

single stem cell that had the ability to undergo at least 130 population doublings (Rochat *et al.,* 1994). Human endometrial epithelial cells were also able to undergo serial cloning more than 3 times and underwent 30 population doublings over four months in culture (Gargett *et al.*, 2009).

1.1.2.4 Differentiation

Differentiation is a change of cellular phenotype mediated by epigenetic change and/or gene expression. Adult stem cells need to generate differentiated progeny of the body organ or tissue in which they reside to maintain homeostasis (Potten & Loeffler, 1990; Gargett, 2007). The differentiation capacity of adult stem cells can be classified into multipotent and unipotent (Slack, 2000). Multipotent differentiation is best described in the haematopoietic system (Weissman, 2000), hair follicle (Fuchs & Segre, 2000) and in the breast where several different mature epithelial daughter cells are produced that constitute the entire epithelial tissue (Stingl, 2005; Smith, 2006). With unipotent differentiation only one type of differentiated cell is produced from a stem cell and as exemplified by spermatogonial stem/progenitor cells (Smith, 2006). In the breast, three different cell types differentiate from a single cell and each cell lineage is characterised by a specific differentiation marker (Stingl, 2005). This was demonstrated by a single cell which reconstituted a whole mammary gland *in vivo*. This single cell produced the luminal and myoepithelial TA cells which then committed to differentiate into functional lobuloalveolar units of a mammary gland (Shackleton *et al.*, 2006).

1.1.3 Stem cell niche

Given that stem cells are quiescent and long-lived to maintain tissue homeostasis, they need to reside in a specialised microenvironment termed the niche (Schofield, 1978).

Extensive research in the *Drosophila* germ stem cells has provided important information about the niche which is common to all mammalian stem cell systems. The niche is a complex environment that provides factors to maintain stem cell function and regulate self-renewal and differentiation cell fate decisions (Eckfeldt *et al.*, 2005; Li & Xie, 2005). The niche comprises cellular and extracellular components, together with secreted factors provided by neighbouring niche cells to regulate cell fate decisions (Fuchs *et al.*, 2004).

The niche microenvironment varies in different tissues, and it regulates stem cells through different signals often using cadherin and integrin interactions. High levels of integrin are indicative of epidermal stem cells, as integrins support stem cell anchoring to the extracellular matrix within the niche (Watt, 2002; Fuchs *et al.*, 2004; Jones & Wagers, 2008). The stem cell niche also plays a protective role for gene function and signalling activity of the resident stem cells (Rizvi & Wong, 2005). Signalling pathways including the Wnt / β-catenin, transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP) are all important regulators of the stem niche cells (Jones & Wagers, 2008). The N-cadherin / β-catenin complex was essential to maintain the haematopoietic stem cell niche (Calvi *et al.*, 2003; Zhang *et al.*, 2003; Li & Xie, 2005). Additionally, the niche also operates via epithelial-mesenchymal interactions, which is important for tissue morphogenesis and differentiation (Blanpain *et al.*, 2007). It is believed that the underlying mesenchymal or stromal cell population residing in the niche are responsible for determining epithelial stem cell fate. Disruption of this interaction could result aberrant genetic or epigenetic changes involving key signalling pathways and uncontrolled cellular proliferation (Beachy, 2004; Goldman, 2007).

1.1.4 Signalling pathways in stem cells

Wnt, Notch, Hedgehog and BMP are all known developmental signalling pathways important for stem cell function (Reya *et al.*, 2001; Blanpain *et al.*, 2007). These pathways directly or indirectly influence stem cell self-renewal, proliferation. Aberrant alterations in signalling pathways can contribute to cancer initiation and un-regulated cell proliferation (Reya & Clevers, 2005; Rizvi & Wong, 2005).

1.1.4.1 Wnt signalling in stem cells

The Wnt signalling pathway is involved in embryogenesis and patterning of the mesodermal lineage. It also regulates stem cell self-renewal and oncogenesis in many adult tissues and organs (Reynold *et al.*, 2009). Disruption to this pathway could result in abnormal growth and cancer development (Logan & Nusse, 2004; Blanpain *et al.*, 2007). The Wnt genes were sequenced based on the homology with *Wnt-1* in the mouse and *wingless (wg) in Drosophila* (Cadigan & Nusse, 1997). Wnt genes encode for secreted cysteine-rich glycoproteins and in mammals, there are 19 Wnt ligands that could initiate downstream signalling cascade (van de Wetering *et al.*, 2002; Blanpain *et al.*, 2007; MacDonald, 2009).

The canonical Wnt signalling pathway concerns the interaction between the Wnt ligands, the surface receptors, Frizzled (Fzd), co-receptor LRP5/6, the central player, β-catenin and its inhibitory complex (Logan & Nusse, 2004; Reya & Clevers, 2005). In the absence of Wnt ligand (Fig. 1.2A), cytoplasmic β-catenin is tightly bound to the destruction complex comprising APC, Axin, GSK-3β and CK1. Subsequently, β-catenin phosphorylation targets it for degradation by the proteosome, thus maintaining a low level of β-catenin in the cytoplasm (Logan & Nusse, 2004; MacDonald, 2009). In the presence of the Wnt ligand (Fig. 1.2B), which binds to the Frizzed (Fzd) receptor and the LRP5/6 co-receptors,

a heterodimer complex forms that recruits the scaffolding protein Dishevelled (Dvl) to sequester the destruction complex to the receptors. Consequently, degradation of β-catenin is inhibited, resulting accumulation of cytoplasmic β-catenin (Fig. 1B). β-catenin is translocated to the nucleus and where it coupled with TCF/LEF complex to activate Wnt target genes (Behrens *et al.*, 1996; Huber *et al.*, 1996; Logan & Nusse, 2004; MacDonald, 2009) (Fig. 12B). β-catenin, a key component of the Wnt pathway also plays an essential role in cell-cell adhesion by interacting with the cadherin superfamily (Hajra & Fearon, 2002). Thus cadherin-mediated adhesion can act as a negative regulator of Wnt signalling by recruiting β-catenin to the cell surface (Rattis *et al.*, 2004).

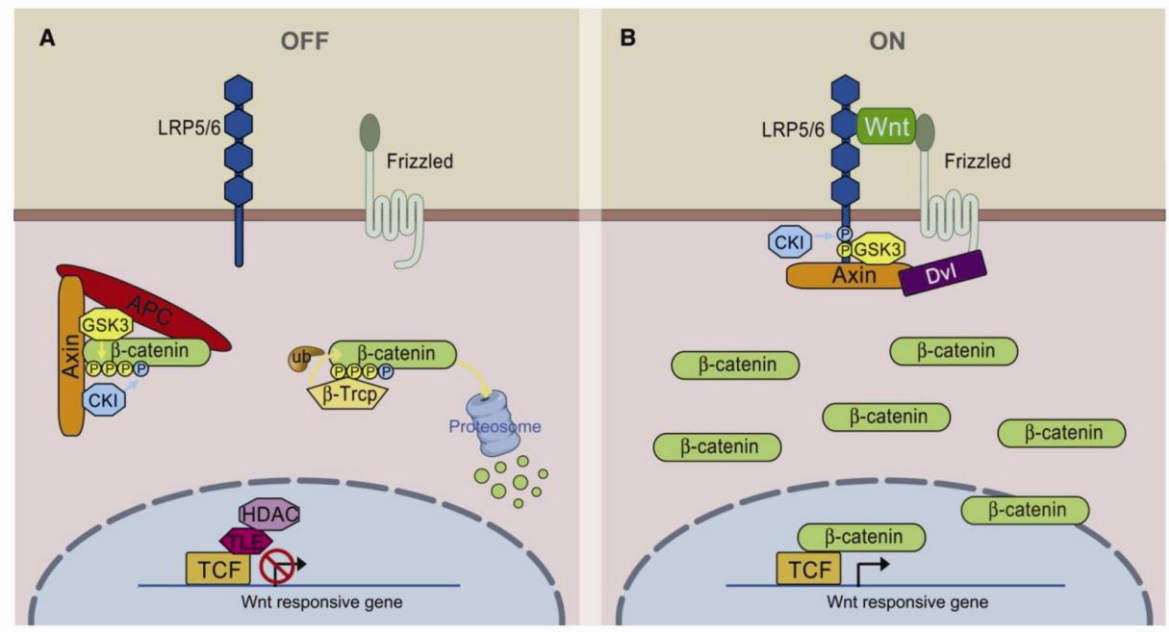


Figure 1.2 Wnt / β-catenin signalling pathway. A) In the absence of Wnt ligand, cytoplasmic β-catenin is targeted for degradation by the destruction complex (APC, Axin, GSK-3β and CK1). **B)** In the presence of Wnt ligand, β-catenin is freed from the destruction complex and translocated to the nucleus where it associates with TCF and activates Wnt target gene expression. Adapted from MacDonald et al (2009) *Developmental Cell* **17**(1): 9-26 with permission from Elsevier. APC: adenomatous polyposis coli, GSK-3β: glycogen synthase kinase-3β, CK1: casein kinase- 1. TCF: T cell factor.

Wnt signalling is involved in the function of nearly all adult stem cells of epithelial tissues (Reya & Clevers, 2005; Blank *et al.*, 2008). The best example of the Wnt / β-catenin

signalling pathway is its control of intestinal stem cell self-renewal and Paneth cell differentiation via TCF4 and the target gene c-MYC. β-catenin coupling with TCF4 is essential for intestinal crypt stem cell self-renewal and disruption to this interaction will result a switch to activate differentiation genes (van de Wetering *et al.*, 2002; Blanpain *et al.*, 2007). Subsequent studies identified intestinal stem cell marker Lgr5, a Wnt target gene, which allows further characterisation of the intestinal stem cell population (van de Wetering *et al.*, 2002; Barker *et al.*, 2007). Similarly, β-catenin/TCF4/LEF complex regulates quiescent hair follicle stem/progenitor cells, since a gain- and loss- of function studies in mice have shown that mice lacking LEF1 do not develop hair follicles (van Genderen *et al.*, 1994; Reya & Clevers, 2005; Blanpain *et al.*, 2007). The Wnt / β-catenin interactions, particularly Wnt 3a and Wnt 5a ligands have been shown to promote self-renewal of mouse haematopoietic stem cells (HSC) *in vitro* and recapitulate the haematopoietic system *in vivo* (Van Den Berg *et al.*, 1998; Murdoch *et al.*, 2003; Reya & Clevers, 2005; Nemeth *et al.*, 2007; Fleming *et al.*, 2008). Additionally, N-cadherin / β-catenin complex is essential for the maintenance of the HSC niche in that N-cadherin promotes HSC to adhere to the osteoclast niche cell (Calvi *et al.*, 2003; Zhang *et al.*, 2003; Li & Xie, 2005).

1.1.4.2 Other signalling pathways in stem cells

Similar to the Wnt signalling pathway, Notch signalling controls selective stem cell fate determination (Blanpain *et al.*, 2007). In the mammal, the Notch family has four transmembrane receptors and signal transduction is carried out by ligand-receptor interactions with the surface ligands Delta and Jagged (Rizvi & Wong, 2005; Blanpain *et al.*, 2007). Notch has the ability to amplify intestinal progenitor cell proliferation and prevent differentiation (Schröder & Gossler, 2002; Fre *et al.*, 2005; Stanger *et al.*, 2005).

Similarly, Notch signalling is also involved in breast stem cell self-renewal producing progenitor cells with a ten fold increase in proliferation capacity (Dontu *et al.*, 2004).

The Hedgehog signalling pathway is another important regulator of stem cell fate. It has 3 ligands (Sonic, Indian and Desert) that binds to the Pached (Ptc) receptor that initiate downstream signalling via Smoothened (Smo) (Taipale & Beachy, 2001). Hedgehog also interacts with the Wnt signalling pathway to regulate self-renewal and differentiation in multiple types of epithelial stem cells (Watt, 2004). In particular, sonic hedgehog (Shh) is essential for hair follicle development and has roles in the neural stem cell niche (St-Jacques *et al.*, 1998; Lai *et al.*, 2003; Machold *et al.*, 2003). Shh and Indian hedgehog (Ihh) have the capacity to maintain mammary epithelial stem cell self-renewal and keep these cells in the undifferentiated state. This was demonstrated by a 3.5 fold increase in the number of mammosphere-derived cells with multi-lineage differentiation. When the cells were treated with the Hedgehog inhibitor, cyclopamine, a 1.8 fold decreased was observed (Liu *et al.*, 2006).

The BMP molecules are members of the TGF-β superfamily and are important during embryogenesis. The BMP signalling pathway controls stem cell self-renewal and maintenance (Rizvi & Wong, 2005). BMP has been shown to inhibit intestinal stem cell self-renewal by suppressing Wnt signalling (He *et al.*, 2004). BMP coupling together with the leukaemia inhibitory factor (LIF) could suppress embryonic stem (ES) cell differentiation and enhance self-renewal for two and three passages in culture (Ying *et al.*, 2003). Furthermore, the presence of BMP allowed multiple passages of undifferentiated ES cells and expressed ES-cell lineage specific markers (Ying *et al.*, 2003).

1.1.5 Markers of stem cells

Given that rare adult stem cells have no distinguishing morphological features from their mature progeny, a major research effort has been undertaken to identify specific markers of adult stem cells (Cai *et al.*, 2004; Díaz-Flores Jr *et al.*, 2006; Gargett, 2007). Markers examined include EpCAM, α_6 integrin, keratin 14, which have identified multiple sub-types of mammary epithelial progenitor cells (Stingl *et al.*, 2001; Visvader & Lindeman, 2008). Similarly, β_1 integrin can be used to isolate human epidermal stem cells (Jones & Watt, 1993), while α_6 integrin / CD71 identified epidermal stem cells and different corneal epithelial stem/progenitor cells populations (Li *et al.*, 1998; Hayashi *et al.*, 2008). Other markers include Lgr5, which identified intestinal stem cells, and is responsible for maintaining their long-term self-renewal stem cells in the intestine, colon and hair follicles (Barker *et al.*, 2008; Barker *et al.*, 2010). Human keratinocyte stem cells, prostate epithelial stem cells and endometrial stromal stem cells have also been isolated using known stem cell makers (Li *et al.*, 1998; Richardson *et al.*, 2004; Schwab & Gargett, 2007; Schwab *et al.*, 2008). The identification of stem cell markers in several organs have proven to be successful in that a stem cell population can be isolated and distinguished from their mature differentiated cells. This has enabled further characterisation of their molecular and cellular phenotypes and understanding their role in tissue maintenance and cancer progression.

1.2 Cancer stem cells

Extensive research has been carried out to find a common mechanism responsible for cancer initiation. Many studies have shown that a small rare population, known as cancer stem cells (CSC) are responsible for tumour formation and maintenance (Reya *et al.*, 2001; Clarke *et al.*, 2006). Tumours are believed to have a similar hierarchy of cells with

functional properties analogous to that of normal tissues. CSC posses self-renewal capacity, proliferative ability to drive tumour expansion and can differentiate into many subtypes comprising the overall bulk of the tumour (Jordan *et al.*, 2006; Hubbard & Gargett, 2010). Cell populations in cancer tissue are heterogeneous with respect to gene profiles, proliferative potential and differentiation capacity, hence the term "tumour heterogeneity" (Visvader & Lindeman, 2008). The cellular mechanism of tumour heterogeneity is unknown and is of much interest as it is thought to involve CSC. One current proposed hypothesis is that CSC originate from normal stem cells by acquiring genetic mutations and/or epigenetic changes over time, especially in tumour suppressor genes resulting in neoplastic transformation (Miller *et al.*, 2005; Jordan *et al.*, 2006) (Fig. 1.3). CSC could arise from progenitor cells acquiring genetic changes conferring the ability to self-renew and also differentiate to form cells comprising the bulk of the tumour (Pardal *et al.*, 2003; Jordan *et al.*, 2006) (Fig 1.3). CSC are resistant to chemotherapy and radiation therapy, enabling them to survive these treatments and re-establish the tumour. CSC can migrate to different sites, hence CSC are responsible for tumour metastasis (Pardal *et al.*, 2003; Clarke & Fuller, 2006; Visvader & Lindeman, 2008).

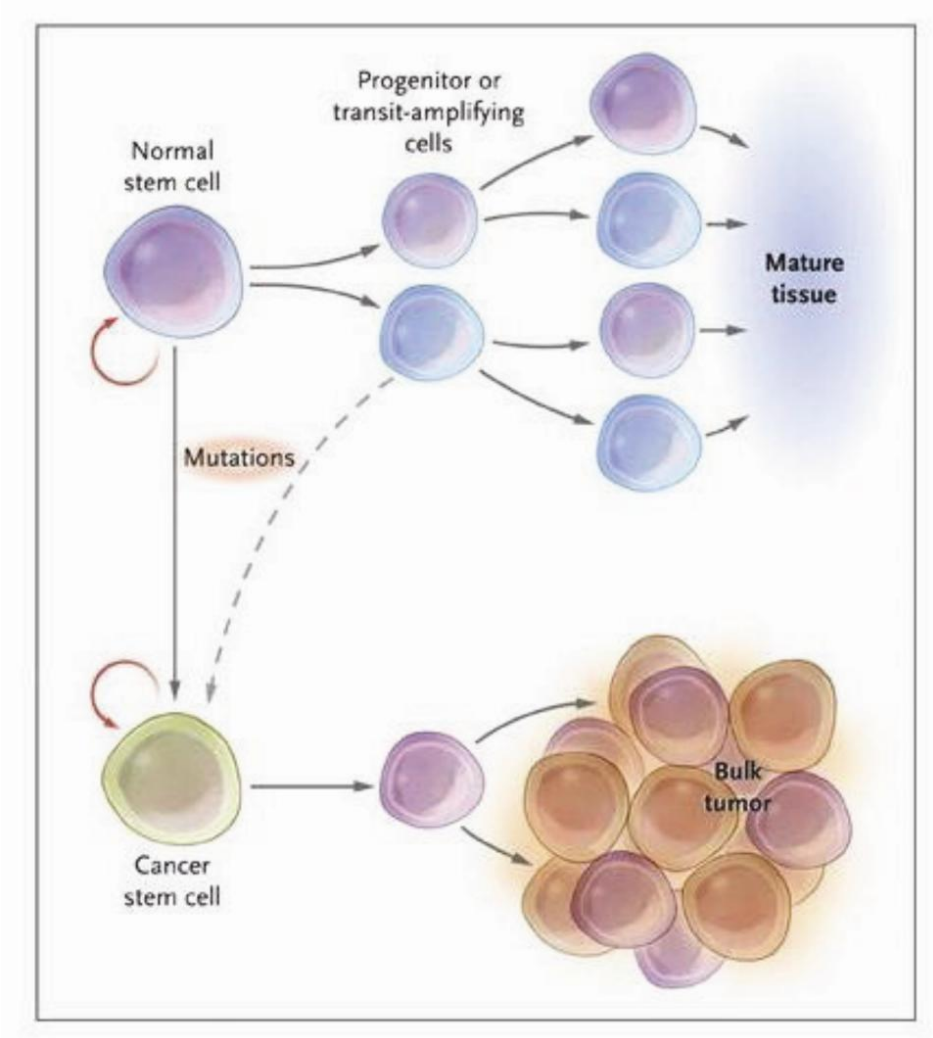


Figure 1.3 Potential source of cancer stem cells. Cancer stem cells exhibit similar properties to normal stem cells with the ability to self-renew, high proliferative potential and differentiation capacity. Cancer stem cells are thought to be derived from normal stem cells, or progenitor cells gaining genetic or epigenetic mutations which subsequently proliferate and differentiate to form the bulk of tumour. Adapted from Jordan et al (2006) *New England Journal of Medicine* 355(12):1253-1261 with permission from the Massachusetts Medical Society.

Additionally, another proposed concept explaining tumour heterogeneity is the clonal evolution model in which a dominant tumour cell population rapidly adapts to the new environment to drive tumour formation (Nowell, 1976; Visvader & Lindeman, 2008). It is likely that multiple tumour cell subtypes comprise the tumour, where each subtype capables of sustaining the tumour. It is also likely that the two concepts are mutually exclusive, thus more research is required to enable appropriate therapeutic treatments

targeting these processes including self-renewal (Adams & Strasser, 2008; Visvader & Lindeman, 2008).

Given that the scope of this thesis is to identify markers of stem cells and cancer stem cells in the endometrium and endometrial cancer, only the cancer stem cell theory will be discussed.

1.2.1 Properties of cancer stem cells

By definition, cancer stem cells possess similar properties to normal stem cells. They have the ability to self-renew to generate additional cancer stem cells, and also the ability to produce phenotypically diverse progeny with limited proliferative potential which differentiate into mature cell types making up the bulk of the tumour (Reya *et al.*, 2001; Pardal *et al.*, 2003; Clarke *et al.*, 2006) (Fig. 1.3). Another feature of CSC is their ability to escape cancer treatments. Chemotherapy and radiation therapy may eradicate the bulk of the tumour, however, the CSC survives the treatment and is subsequently responsible for tumour regrowth and patient relapse (Miller *et al.*, 2005; Jordan *et al.*, 2006).

1.2.2 Evidence of cancer stem cells

The first evidence of CSC was obtained in the context of acute myeloid leukaemia (AML) in which a rare subset of cells (0.01 – 1%), distinct from the remaining AML cells were able to induce leukaemia when transplanted into immunocompromised mice (Lapidot *et al.*, 1994; Bonnet & Dick, 1997). This concept has been extended to the identification of CSC in other solid tumours including the breast, brain, colon cancer and endometrial cancer (Al-Hajj *et al.*, 2003; Singh *et al.*, 2003; O'Brien *et al.*, 2007; Ricci-Vitiani *et al.*, 2007; Hubbard *et al.*, 2009).

1.2.3 Marker of cancer stem cells

Mounting data in the last decade has shown that combinations of surface makers and immunosorting (flow cytometry and/or magnetic bead sorting) are useful to prospectively isolate rare stem cells from some epithelial tissues. Some surface markers including CD44, CD24 and CD133 have been found common to several cancers (Table 1.1). Evidence of CSC was first demonstrated in breast cancer (Al-Hajj *et al.,* 2003). As few as 100 of the $CD44^{+}CD24^{-/low}$ cell sub-population generated tumours when transplanted into mice, indicating CSC enrichment for tumour-initiating capacity (Al-Hajj *et al.,* 2003). Interestingly, as few as 0.2% of $CD44^{+}CD24^{+}$ of total pancreatic cancer cells enriched for pancreatic cancer-initiating cells (Li *et al.*, 2007).

Similar results have been observed in brain cancer where $CD133^{+}$ cells generated spheres *in vitro* which exhibit self-renewal and high proliferative potential (Singh *et al.*, 2003). $CD133^{+}$ cells were also isolated colon cancer-initiating cells with 200 fold enrichment that were capable of forming tumours resembling original parent tumour *in vivo* (O'Brien *et al.*, 2007; Ricci-Vitiani *et al.,* 2007). In contrast, work from another laboratory claimed that colon cancer stem cells were enriched in the $EpCAM^{hi/+}CD44^{+}$ sub-populations and that this population has very little overlap with $CD133^{+}$ cells (Dalerba *et al.*, 2007). Additionally, $CD44^{+}\alpha_2\beta_1{}^{hi}CD133^{+}$ sub-populations identified prostate cancer stem cells (0.1%) (Collins *et al.,* 2005) while $CD44^{+}CD117^{+}$ can give rise to ovarian cancer (Zhang, 2008).

TABLE 1.1 Common stem cell surface markers identifying human cancer stem cells in some organs/tissues

Stem cell markers	Organs/ Cancer types	References
$CD44^{+}CD24^{-/low}$	Breast cancer	Al-Hajj et al 2003
$CD44^{+}CD24^{+}EpCAM^{+}$	Pancreatic cancer	Li et al 2007
$CD44^{+}$	Head and neck cancer	Prince et al 2007
$CD44^{+}$	Gastric cancer	Takaishi et al 2009
$CD44^{+}$	Prostate cancer	Patrawala et al 2006
$CD44^{+}\alpha2\beta1^{hi}CD133^{+}$	Prostate cancer	Collins et al 2005
$EpCAM^{+}CD44^{+}$	Colon cancer	Dalerba et al 2007
$CD44^{+}CD117^{+}$	Ovarian cancer	Zhang et al 2008
$CD24^{+}$	Ovarian cancer	Gao et al 2010
$CD133^{+}$	Brain cancer	Singh et al 2003
$CD133^{+}$	Colon cancer	O'Brien et al 2007, Ricci-Vitini et al 2007

1.2.4 Prospective isolation of cancer stem cells

Normal stem cells and cancer stem cells share similar properties, however there are no morphological features distinguishing them from their mature progeny. It is imperative to identify surface markers that select for the stem cell population. These markers can be used to examine stem cell functional properties both *in vitro* and *in vivo* (Fig. 1.4). All techniques discussed below are applicable to the prospective isolation of both normal and cancer stem cells. Serial passage of colony-forming cells is used to measure the proliferative potential of progenitor cells *in vitro* (Fig. 1.4A). Sphere formation is another *in vitro* technique frequently used to determine stem cells. Stem cells are expect to grow as floating colonies in anchorage independent manner (Dontu *et al.*, 2003; Singh *et al.*, 2003) (Fig. 1.4B). Side population (SP) is defined as the ability of a rare stem cell subset to efflux a DNA binding dye (Hoechst 33342) using the ATP binding cassette transporter G2 (ABCG2). This technique was first demonstrated in the haematopoietic system (Goodell *et*

al., 1996). SP cells have been used widely to identify rare stem cells in many body organs systems (Alvi *et al.*, 2003; Montanaro *et al.*, 2004; Kato *et al.*, 2007) and cancers (Patrawala *et al.*, 2005; Friel *et al.*, 2008).

In vivo serial transplantation is the gold standard method to measure normal and cancer stem cell function by determining the ability of cellular growth and expansion from a limiting number of transplanted cells (Visvader & Lindeman, 2008; Hubbard & Gargett, 2010) (Fig. 1.4C). Another *in vivo* approach to identify stem cells is to investigate their quiescence state. Label-retaining cells (LRC) technique relies on nuclear labelling with a DNA synthesis dye, bromodeoxyuridine (BrdU) followed by a chased period of time to discriminate between stem cells, TA cells and the mature differentiated cells (Gargett, 2007). Slow-cycling, quiescent stem cells will retain the BrdU dye while the rapidly-cycling TA cells will dilute the label to an undetectable level as they progress through each cell division. LRC have been detected in mammnay gland, epidermis and prostate and their stem cell properties demonstrated (Morris *et al.*, 1986; Morris & Potten, 1994; Tsujimura *et al.*, 2002; Welm *et al.*, 2002). These methods are mostly used by researchers in the search for identifying normal and cancer stem cell markers.

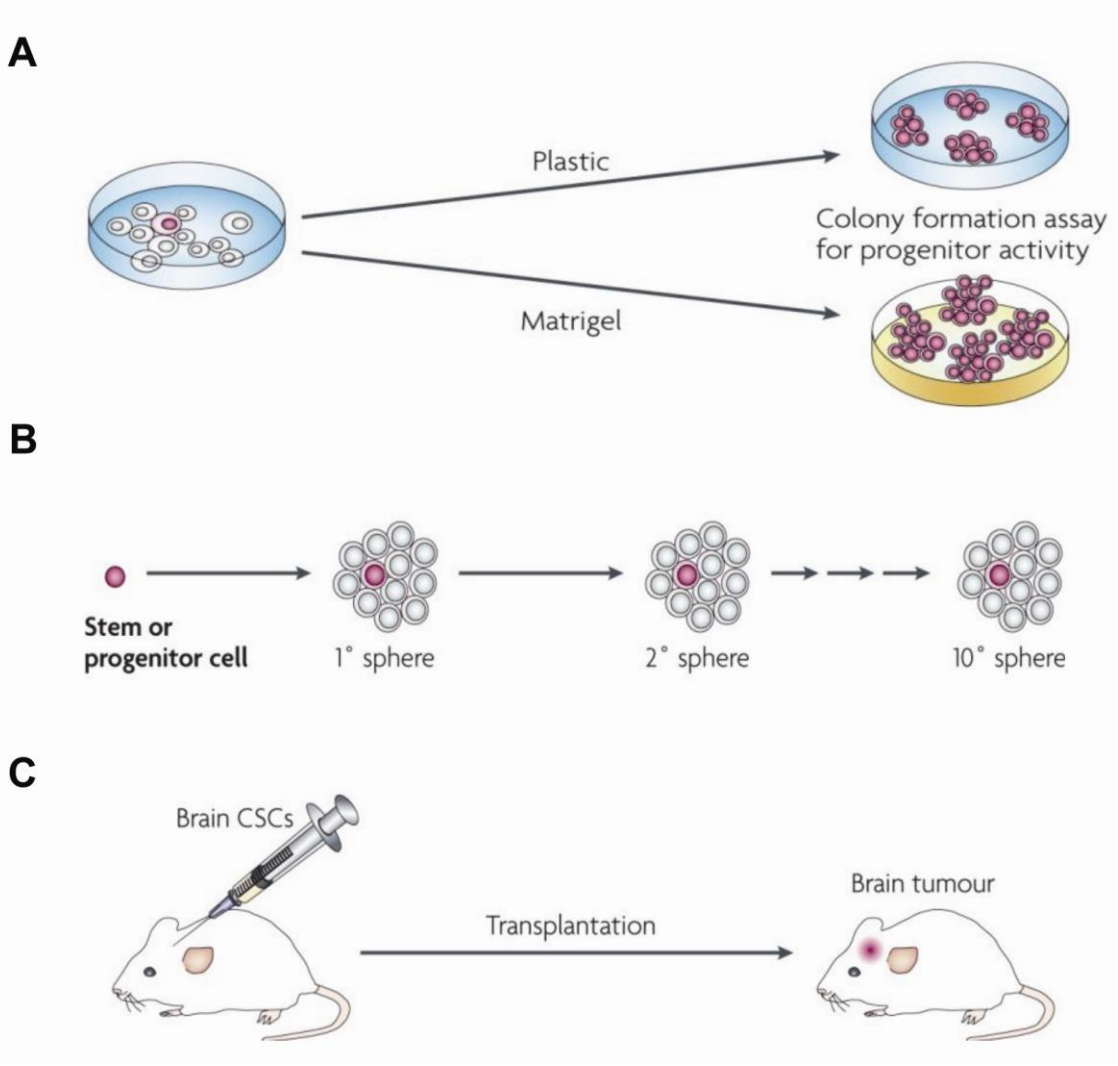


Figure 1.4 *In vitro* and *in vivo* assays of stem/progenitor cells. A) Cell population of interest is seeded into culture plates (plastic or Matrigel basement membrane) and examine for colony-forming unit activity. Serial passage of CFU is a read out of proliferative potential of progenitor cells. **B)** In vitro propagation of spheres which predicts the CSC existence. **C)** The gold standard method of serial transplantation of sub-population of interest into immunocompromised mice, and evaluate self-renewal of stem cells or cancer stem cells. Adapted from Visvader & Lindeman (2008) *Nature Reviews Cancer,* 8 (10):755-768 with permission from Nature Publishing Group.

1.2.5 Heterogeneity and frequency of cancer stem cells

While surface markers are useful for the prospective isolation of CSC population, combination of several different markers provides further enrichment for CSC of the same cancer. For example, $CD133^{+}$ and $EpCAM^{hi/+}CD44^{+}$ were both reported as markers enriching for colon cancer stem cells (Dalerba *et al.*, 2007; O'Brien *et al.*, 2007; Ricci-

Vitiani *et al.*, 2007). However, there is little overlap between the sub-populations (Visvader & Lindeman, 2008). These findings suggest that several CSC phenotypes may exist in the same cancer type which are capable of driving tumour expansion. Alternatively, it is possible that CSC express multiple markers that can be used to isolate them. Additionally, the frequency of tumour formation also varied between each of the sub-populations and between cancer types, ranging from one single cell to 200 cells to >2000 cells (Adams & Strasser, 2008; Visvader & Lindeman, 2008). This further implies that more research is needed to improve or re-define markers for the isolation of cancer stem cells.

1.2.6 Signalling pathways in cancer stem cells

Signalling pathways that maintain normal stem cell function are also involved in cancer stem cell regulation, albeit they are deregulated (Visvader & Lindeman, 2008). The Wnt signalling pathway is the most commonly studied for its involvement in both normal stem cell development and cancer and is best described in the intestinal epithelium (Logan & Nusse, 2004; Reya & Clevers, 2005) (Section 1.1.4). Wnt signalling is required for self-renewal of the intestinal epithelial stem cells and to maintain their niche. Mutations that activate the Wnt signalling pathway lead to hyperproliferation of the crypt progenitor cells, resulting benign polyps (Pardal *et al.*, 2003; Reya & Clevers, 2005; Visvader & Lindeman, 2008). Similarly, mutations in the Wnt inhibitor complex (APC or Axin2) also lead to abnormal downstream effects on β-catenin and hence colon cancer (Taipale & Beachy, 2001; Reya & Clevers, 2005). Wnt signalling also regulates colon CSC (Vermeulen *et al.*, 2010) and self-renewal of chronic myeloid leukaemia stem cells (Zhao *et al.*, 2007). Further, aberrant activation of the Wnt signalling pathway is also responsible for endometrial cancer (Fukuchi *et al.*, 1998; Moreno-Bueno *et al.*, 2002; Wang *et al.*, 2010).

Notch signalling is well described for its role in the development of breast stem/progenitor cells (Dontu *et al.*, 2004). Recent work has shown that aberrant Notch activation leads to breast cancer (Stylianou *et al.*, 2006). A subsequent study demonstrated that Notch4 signalling activity was eight fold higher in the $CD44^{+}CD24^{-/low}$ breast CSC population. Consequently, inhibition of Notch4 receptor reduced breast CSC activity *in vitro* and reduced tumour formation *in vivo* (Harrison *et al.*, 2010).

Hedgehog signalling is also well studied for its involvement in normal and cancer stem cell function in the setting of hair follicle progenitor cells. When overexpression occurs, basal cell carcinoma resulted (Oro *et al.*, 1997; Taipale & Beachy, 2001; Pardal *et al.*, 2003). Hedgehog signalling is also involved in multiple myeloma, where it induces clonal expansion of the progenitor and transit-amplifying cells (Peacock *et al.*, 2007). Other signalling networks including BMI-1 *(a gene of the Polycomb family thought to regulate stem cell self-renewal and proliferative activity)* and PTEN *(a tumour suppressor gene)*, are also playing key roles in controlling stem cell self-renewal and cancer cell proliferation (Pardal *et al.*, 2005).

1.2.7 Therapeutic implications

Given that some common signalling pathways are shared by normal stem cells and cancer stem cells, suggesting they may be a cell of interest as a therapeutic target. However, important consideration is that cancer treatment should not have an irreversible effect on the normal stem cells (Pardal *et al.*, 2003; Visvader & Lindeman, 2008). In addition, cancer stem cells are rare, quiescent and known to escape conventional therapies, therefore understanding the response of cancer stem cells to treatments is absolute essential to enable their eradication (Miller *et al.*, 2005; Klonisch *et al.*, 2008; Visvader & Lindeman, 2008). Currently, in some cancers, drugs are being developed and trialed (Curtin & Lorenzi, 2010;

de Sousa *et al.*, 2011), but targeting the rare CSC population is still a challenge. Thus, identifying candidate surface markers is imperative for the prospective isolation of normal stem cells and their cancer stem cell counterpart. It also enables our understanding of stem cell function in tissue homeostasis and in the abnormal setting when cancer stem cells drive tumour expansion. Only when these markers are identified will it be possible to improve therapeutic outcomes based on eradicating CSC.

1.3 Human endometrium

1.3.1 Structure and function

The human endometrium or the mucosal lining of the uterus is a unique tissue that undergoes cyclical processes of regeneration, differentiation and shedding throughout the menstrual cycle of reproductive aged women in response to cyclical changes of ovarian steroid hormones estrogen and progesterone (Jabbour *et al.*, 2006; Gargett *et al.*, 2008). The endometrium consists of two main layers, the upper functionalis layer and the lower basalis layer (Figure 1.5A, B). Many cell types comprise the endometrium including the luminal and glandular epithelial cells, stromal cells, endothelial cells, vascular smooth muscle cells and other immune competent cells (Buffet *et al.*, 1998).

The main function of the endometrium is to provide a suitable environment for embryo implantation and to support and nurture the foetus throughout gestation. However, in the absence of implantation, the upper functionalis layer is shed and remodelling occurs in preparation for the next cycle (Dockery, 2002).

1.3.2 Zonation of the endometrium

Traditionally, the endometrium is divided into two main layers according to histological features and functional differences (Fig. 1.5). However, based on studies of the primate uterus, the endometrium could be divided further, into four zones based on the histological appearance of the glands (Padykula *et al.*, 1984). Functionalis zone 1, the compact zone consists of the surface luminal epithelium. Functionalis zone 2, spongy zone contains loosely packed stroma surrounding the upper third of the glands (Uduwela *et al.*, 2000; Strauss III & Lessey, 2009). Basalis zone 3 contains densely packed stroma, supporting vasculature and the branching middle third of the glands. Basalis zone 4 comprises dense stroma and the basal regions of the glands that extend to the adjacent myometrium (Uduwela *et al.*, 2000; Strauss III & Lessey, 2009) (Fig. 1.5).

The functionalis undergoes extensive monthly remodelling in response to estrogen and progesterone and is shed during menstruation (Uduwela *et al.*, 2000; Jabbour *et al.*, 2006). In contrast, the basalis is relatively stable throughout the menstrual cycle and is responsible for the regeneration of the new functionalis (McLennan & Rydell, 1965; Jabbour *et al.*, 2006). Variations in proliferation rates between the zones are supportive of the quadripartite zonation of the endometrium (Padykula *et al.*, 1984; Padykula *et al.*, 1989). In fact, the highest proliferation rates were in the upper third of the functionalis while rates remained relatively low the basalis throughout the menstrual cycle (Ferenczy *et al.*, 1979; Okulicz *et al.*, 1997; Okulicz, 2002). Different patterns of cell proliferation, programmed cell death and gene expression also evident across the two layers (Strauss III & Lessey, 2009).

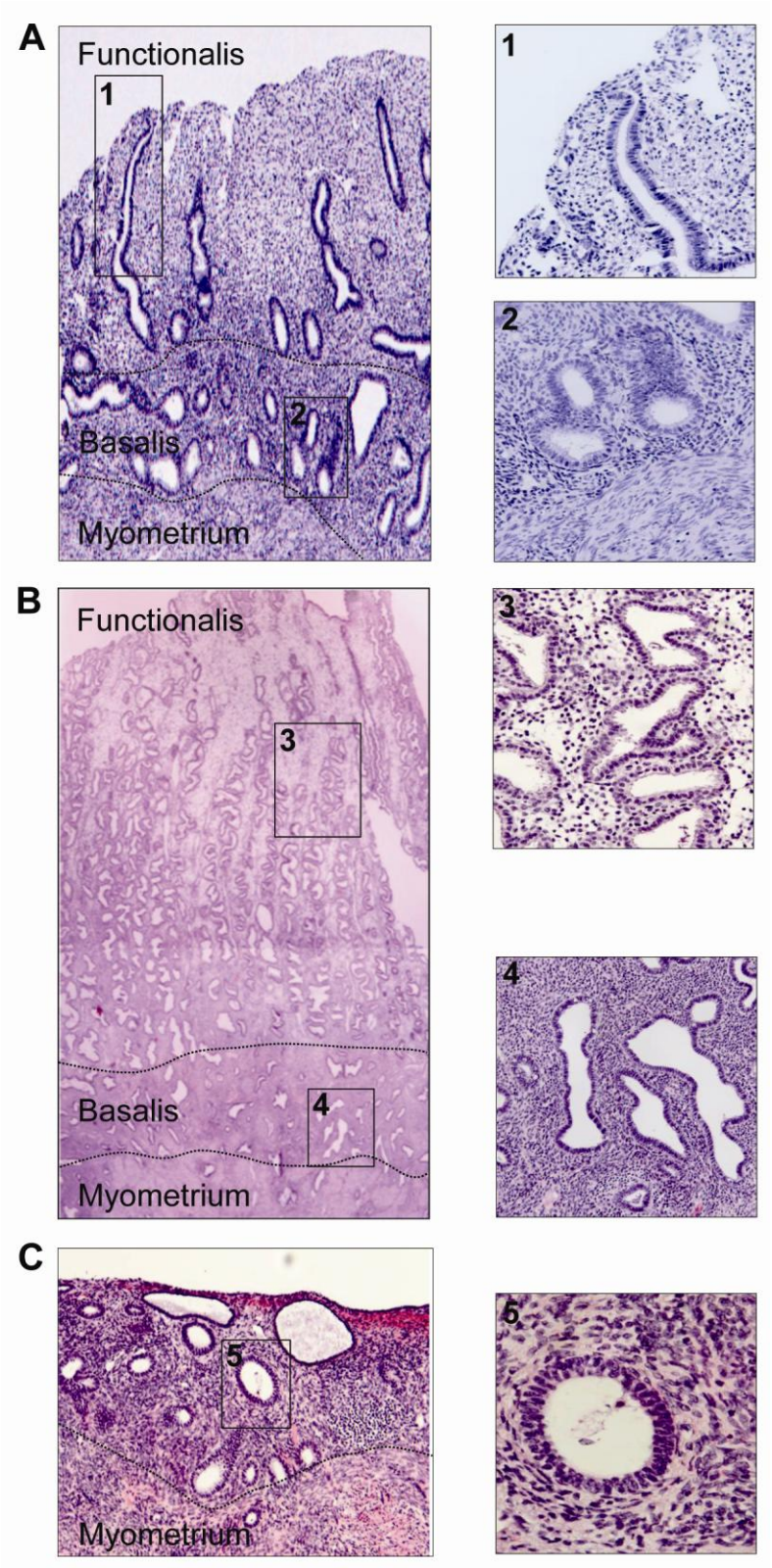


Figure 1.5 The human endometrium. Full thickness **A)** proliferative and **B)** secretory pre-menopausal endometrium consists of multiple cell types including epithelial cells in the upper functionalis (**1, 3**) and the basalis (**2, 4**) layer. **C)** Post-menopausal endometrium is thin and basalis-like.

1.3.3 Post-menopausal endometrium

The endometrium of post-menopausal women is thin, atrophic and consists of a few glands and scattered stroma (Klaassens *et al.*, 2006; Gargett, 2007; Strauss III & Lessey, 2009; Kurman *et al.*, 2011) (Figure 1.5C). Once circulating hormones diminish, the functionalis is virtually absent and mitotic activity ceases. Epithelial cells shrink and stroma becomes fibrotic (Wise *et al.*, 2005; Strauss III & Lessey, 2009). The exact date of menopause cannot be determined; however, it is known to occur spontaneously around the age of 51 (Wise *et al.*, 2005).

Although there is a lack of sufficient estrogen to stimulate post-menopausal endometrial proliferation, synthesis of estrogen receptors is maintained in post-menopausal endometrium (Press *et al.*, 1984; Ferenczy & Bergeron, 1991). As a result, post-menopausal endometrium regenerates to a proliferative type endometrium when estrogen only hormone replacement therapy is given (Ferenczy & Bergeron, 1991), suggesting the presence of stem/progenitor cells. Further evidence indicative of a reservoir of stem cells existing in atrophic endometrium is that gland diversity was not reduced after menopause (Kim *et al.*, 2005; Gargett, 2007). Additionally, hormone treated post-menopausal endometrium has similar morphological features to that of proliferative stage pre-menopausal endometrium (Deligdisch, 2000) with increased mitotic activity and proliferation rates (Chow *et al.*, 2001; Klaassens *et al.*, 2006; Punyadeera *et al.*, 2008). In fact, the immense regenerative capacity of the post-menopausal endometrium was confirmed when it supported pregnancy to term in a 64 year old woman treated by donor in vitro fertilisation (Paulson *et al.*, 2002).

While life expectancy has increased during the last 100 years, the age of menopause has remained unchanged, consequently many women will spend over one-third of their lives in

the menopausal state (Wise *et al.*, 2005). Despite this, there is very little research into understanding the biology of post-menopausal endometrium. Most studies focus on hormone replacement therapy with different combinations of estrogens and progestagens. More specifically, there are no studies comparing endometrial basalis epithelial cells from pre-menopausal women with post-menopausal epithelial cells. Neither have there been any studies that have examined in detail whether these epithelial cells are functionally similar in their response to estrogen to induce cellular proliferation and tissue regeneration. Such investigations will aid in our understanding about the contribution of epithelial cells in endometrial regeneration enhancing our knowledge on the biology of post-menopausal endometrium, the aging process, regeneration and possibly the cause of gynaecological diseases, particularly hyperplasia and endometrial cancer.

1.3.4 Endometrial epithelium

Ontogenetically the endometrium is derived from the Müllerian duct (also known as the paramesonephric ducts), a mesodermally derived tissue as first described in 1825 by Muller, J. (Cunha *et al.*, 2002). The Müllerian epithelium derives from the invagination of the coelomic epithelium and differentiates into simple columnar luminal. In human, glandular epithelium derives from the invagination of the luminal epithelium at day 140 gestation (Kurita *et al.*, 2001). This is followed by the invagination of the luminal epithelium into the stroma and extension of tubular glands through the stroma towards the myometrium (Spencer *et al.*, 2005). From birth to the onset of puberty, the endometrial develops slowly and by six years of age, the glands extend half way to the myometrium, the process continues until puberty when mature endometrial architecture is seen (Gray *et al.*, 2001; Spencer *et al.*, 2005).

Endometrial epithelium is a monolayer of cuboidal to columnar cells covering the interior lining of the uterus (Fig. 1.5). Being a reproductive tract barrier, it provides constant protection against pathogens and external stimuli gaining access to the uterine cavity (Simon *et al.*, 2002). More importantly, it also serves as the site for embryo implantation. The luminal epithelium of the surface lining is important at the time of implantation as it is the first maternal contact point with the blastocyst.

Luminal epithelium consists of ciliated and non-ciliated cells and the ratio varies across the menstrual cycle in line with hormonal fluctuations (Dockery, 2002). At the time of receptivity, the luminal surface of luminal epithelial cells transforms into sac-like protrusions known as pinopodes (Simon *et al.*, 2002; Strauss III & Lessey, 2009). Pinopodes formation is progesterone dependent and is essential for human embryo implantation. This is supported by *in vitro* evidence showing that human blastocyst would only implant on human endometrial epithelial surface at the site where pinopodes were presence (Lopata *et al.*, 2002; Strauss III & Lessey, 2009).

Glandular epithelial cells change from low to tall columnar to pseudostratified appearance as the cycle progress from early to late proliferative phase in response to increasing estrogen levels (Fig. 1.5). They contained small, oval-shaped chromatin-dense nuclei in a sparse cytoplasm, marked by increasing mitotic figures (Ferenczy & Bergeron, 1991; Dockery, 2002; Dallenbach-Hellweg *et al.*, 2010). In the secretory phase, the epithelial glands become increasingly tortuous as they are actively involved in the production and secretion of secretory products known as histotroph (Spencer *et al.*, 2011).

1.3.5 The menstrual cycle

The menstrual cycle is defined as the morphological changes occurring in the endometrium in response to fluctuating levels of ovarian estrogen and progesterone hormones. A complete menstrual cycle is about 28 days and ovulation occurs around day 14. The cycle is divided into three main phases: proliferative, secretory and menstrual (Fig. 1.6) (Dockery, 2002; Jabbour *et al.*, 2006).

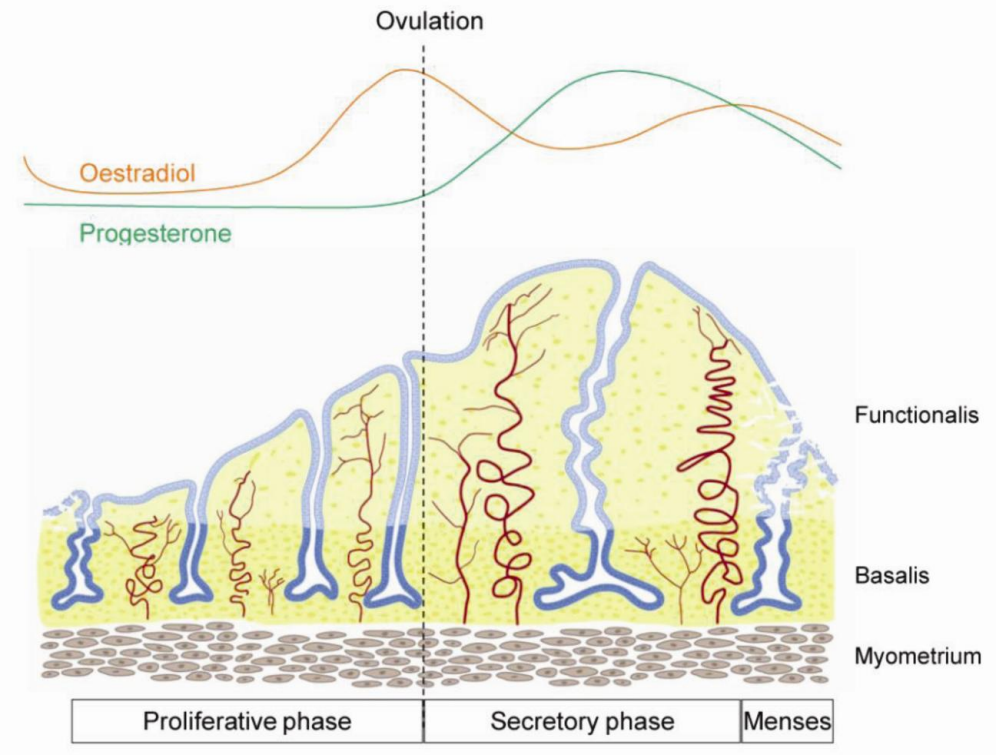


Figure 1.6 Schematic of the human menstrual cycle and the effects of estrogen and progesterone on the morphology of the endometrium. Adapted from Gargett et al (2008) *Molecular and Cellular Endocrinology,* 288 (1-2) 22-29 with permission from Elsevier.

1.3.5.1 Proliferative phase

The proliferative phase follows menstruation and is controlled primarily by estrogen. As estrogen levels rise, cellular proliferation occur in epithelial, stromal and vascular compartments, resulting a substantial 4 – 7 mm of endometrial tissue growth within 4 – 10

days (McLennan & Rydell, 1965). In the early proliferative phase (days 4 – 7), epithelial glands are narrow. They elongate and are lined with pseudostratified cuboidal and low columnar cells and mitotic figures are frequently observed (Dockery, 2002). The nuclear to cytoplasmic ratio is high. In mid- (days 8 – 10) and late- (days 11 – 13) proliferative phase, the endometrium thickens, glandular epithelium appear more pseudostratified, glands become convoluted and tortuous, with more frequent mitosis in the epithelium compared to stroma (Strauss III & Lessey, 2009).

1.3.5.2 Secretory phase

Following ovulation, the secretory phase is dominated by progesterone hormone which stimulates marked epithelial differentiation and secretions, stromal maturation and vascular differentiation (Dallenbach-Hellweg & Poulsen, 1996; Dockery, 2002). In the early secretory phase (days 16 – 19), mitosis figures are much less prominent, nuclear to cytoplasmic ratio is reduced and epithelial glands change from pseudostratified to columnar with glycogen-rich vacuoles pushing the nuclei towards the luminal surface. In mid-secretory phase (days 20 – 22), increasing glycoprotein and polysaccharide secretions are seen in glandular epithelial cells. The stroma become more oedematous and vascularisation is prominent (Dallenbach-Hellweg & Poulsen, 1996; Smith, 2002). Late secretory phase (days 23 – 27) is marked by stromal cell pre-decidualisation beginning around the spiral arterioles and extending to the stroma underneath surface epithelium. The decidual cells are terminally differentiated as are the epithelial cells of the functionalis. Glandular secretions have reached a maximum and glandular epithelial cells become serrated and tortuous and apoptotic activity is visible (Buffet *et al.*, 1998; Kurman *et al.*, 2011).

1.3.5.3 Menstruation

Menstruation is marked by the shedding of the functionalis layer and is governed by the withdrawal of progesterone (and estrogen) which occurs in the absence of embryo implantation (Salamonsen, 2003; Jabbour *et al.*, 2006). The menstrual phase varies between 3 – 4 days and is characterised of leukocytes infiltration which release of extracellular matrix degradation enzymes, resulting breakdown of glands and stroma with vasculature elements (Dockery, 2002; Smith, 2002). Apoptosis occur in the functionalis epithelium to remove senescent, differentiated cells, resulting a clear demarcation of the basal regions which remains intact (Dallenbach-Hellweg & Poulsen, 1996; Strauss III & Lessey, 2009).

1.3.5.4 Regeneration

After 24 – 48 hours of menstrual bleeding during which time the functionalis layer is shed to exposed a denuded surface of the basalis layer, containing glands, ruptured blood vessels and stroma (Fig. 1.7A) (McLennan & Rydell, 1965; Ludwig *et al.*, 1990; Smith, 2002). The human endometrium is unique in that there is no scarring during the endometrial repair process (Ferenczy, 1976a; Ludwig & Metzger, 1976; Salamonsen, 2003). Scanning electron microscopy analysis of regenerating endometrium revealed that restoration of the surface epithelium begins with proliferation and migration of epithelial cells from the remaining gland stumps (Fig. 1.7B) (Ferenczy, 1976b; Ludwig *et al.*, 1990). Re-epithelisation begins from day 1 with progressive and simultaneous epithelial outgrowth of the free ends of the basal glands in the denuded basalis layer (Fig. 1.7A). By day 3, a low-columnar epithelium covering a large part of the denuded surface becomes evident (Fig. 1.7B) which followed by complete cover of the lining surface epithelium on day four (Fig. 1.7C). The mid-cycle endometrial surface is covered with homogenous

epithelial layer which is only interrupted by the mouth of the glands opening (Fig. 1.7D) (Ferenczy, 1976b; Ludwig *et al.*, 1990).

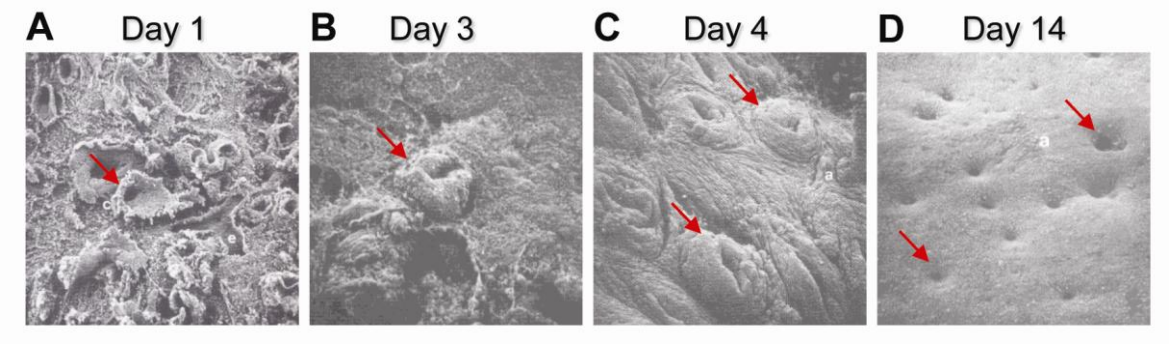


Figure 1.7 Scanning electron microscopy of endometrial epithelial remodelling. A) Day 1, 24 hr after the onset of menstruation, the gland stumps stand out from the surface level of the menstrual wound. **B)** Day 3, epithelial cells migrate out of gland and commence re-epithelialising the surface. **C)** Day 4, formation of micropolyps as the proliferating process continues around the gland openings within the lining surface epithelium. **D)** Day 14, mid-cycle endometrial surface showing homogenous epithelial layer which is only interrupted by gland openings. Red arrow shows glands. Adapted from Ludwig et al (1990) with permission from Cambridge University Press.

The exact mechanism of endometrial regeneration is unclear, but it is widely accepted that glandular regeneration originates from remaining gland stumps in the basalis. Stromal mitosis and expansion follows re-epithelialisation (Novak & Linde, 1924; Ludwig & Metzger, 1976). Given that epithelial mitoses are rare or absent from the basalis, it is suggested that stroma may be involved in epithelialisation of the endometrial surface (Baggish *et al.*, 1967; Garry *et al.*, 2009). However, epithelial mitoses are common during the proliferative phase when estrogen levels are rising, thus it is likely that stromal cells provide paracrine factors enabling epithelial regeneration and glandular growth (Okulicz, 2002). Additionally, it is likely that epithelial stem cells reside in the basalis which are responsible for endometrial regeneration (Prianishnikov, 1978; Padykula *et al.*, 1984; Padykula *et al.*, 1989; Padykula, 1991; Okulicz, 2002; Gargett, 2007).

1.3.6 Hormone regulation of endometrial regeneration

Estrogen is the steroid hormone responsible for endometrial cell proliferation during the proliferative phase while progesterone induces endometrial differentiation in the secretory phase (Smith, 2002; Jabbour *et al.*, 2006). There are two estrogen receptors subtypes; ERα and ERβ. The expression and distribution of ER varies across the menstrual cycle, and correlates with the level of circulating estrogen and progesterone. In human, ER is highest in the estrogen-primed proliferative phase (Barile *et al.*, 1979; Press *et al.*, 1984; Moutsatsou & Sekeris, 1997). ERα is expressed in both glandular epithelial cells and in stroma in the proliferative phase and its expression is down-regulated in the functionalis compared to basalis as the cycle progresses from early- to mid- and late- secretory phase (Press *et al.*, 1984; Matsuzaki *et al.*, 1999; Leyendecker *et al.*, 2002). In the endometrium, ERα is the dominant receptor mediating estrogen stimulated epithelial cell proliferation (Okulicz, 2002). Low level of ERβ expression is localised to majority of glandular epithelial cells and stroma during the proliferative phase. ERβ decreasing dramatically in the secretory stage where the level is undetectable in functionalis and weak in basalis (Matsuzaki *et al.*, 1999). In post-menopausal endometrium, ER is localised to both glandular and stromal cells and could assist in the regeneration process in response to estrogen (Press *et al.*, 1984).

There are at least 3 isoforms of progesterone receptor (PR-A, PR-B and PR-C). PR-A and PR-B are the main receptors that mediate progesterone responses. PR-C is the truncated isoform that may act as a repressor of PR-A and PR-B activity (Smith, 2002; Jabbour *et al.*, 2006). PR-A is the predominant form, specifically in the stroma (Snijders *et al.*, 1992; Wang *et al.*, 1998) (Mote *et al.*, 2000) and is regulated by estrogen (Bethea & Widmann, 1998). PR expression in the glands is highest at mid-late proliferative phase (Lessey *et al.*,

1988). PR-A and PR-B expression declines in functionalis glands but not stroma during the transition from proliferative to secretory phase, while PR distribution in glands and stroma remain unchanged in the basalis (Snijders *et al.*, 1992; Jabbour *et al.*, 2006). Progesterone induces cellular differentiation following estrogen priming of the epithelial cells (Jabbour *et al.*, 2006). While estrogen and progesterone and their receptors are well known regulators orchestrating morphological and cellular changes of the endometrial cycle, the exact signalling mechanism is still unknown and requires further studies.

1.3.7 Gene expression in the endometrium

The human endometrium is a dynamic remodelling tissue that undergoes rounds of cellular proliferation and differentiation in response to sequential signals from circulating ovarian hormones estrogen and progesterone. The dramatic changes in growth, secretion and cell death have been studied at the molecular level with advanced technologies such as global gene expression profiling (Giudice, 2004; Giudice *et al.*, 2008). This is demonstrated by two independent gene expression studies of the human endometrium across the menstrual cycle (Ponnampalam *et al.*, 2004; Talbi *et al.*, 2006). Stage-specific genes of proliferative, secretory and during the window of implantation have also been studied (Carson *et al.*, 2002; Kao *et al.*, 2002; Riesewijk *et al.*, 2003; Horcajadas *et al.*, 2004; Mirkin *et al.*, 2005; Punyadeera *et al.*, 2005).

A whole genome (54,600 genes), high density oligonucleotide microarray study (Talbi *et al.*, 2006) revealed four distinctive gene clusters that were consistent with histological features of proliferative (P), early-secretory (ES), mid-secretory (MS) and late-secretory (LS) endometrium. Subsequent hierarchical clustering produced two main branches where the first branch comprised P and ES and the second branch contained MS and LS clusters (Giudice, 2006; Talbi *et al.*, 2006). Global gene expression profiles can also predict

different stages of the endometrial menstrual cycle (Ponnampalam *et al.*, 2004). This was evident by the identification of seven gene clusters across the menstrual cycle using cDNA microarray (Ponnampalam *et al.*, 2004). These studies highlight this powerful technology has in determining menstrual cycle phases, revealing unique gene signatures that are stage-specific. However, these studies have utilised biopsied endometrium and are unable to dissect out gene profile differences between functionalis and basalis endometrium. Neither do they address cell specific (epithelial and stromal) gene profiles, as the cell types were not separated.

The endometrium is receptive to embryo implantation, thus much attention has been focussed on molecular phenotypes of the window of implantation (Kao *et al.*, 2002; Mirkin *et al.*, 2005). Additionally, the dynamic nature of the endometrium has also prompted studies of estrogen-responsive and progesterone-regulated genes by using samples from the proliferative (Punyadeera *et al.*, 2005; Yanaihara *et al.*, 2005) and secretory phase (Carson *et al.*, 2002; Riesewijk *et al.*, 2003; Horcajadas *et al.*, 2004).

Despite different study designs and data analyses from different studies, biological processes regulated by estrogen and progesterone hormones were found common in these studies. Genes that are relevant to the estrogen-primed proliferative phase include cell proliferation, cell division, cell cycle regulation, DNA synthesis, angiogenesis, cell adhesion molecules (Ponnampalam *et al.*, 2004; Yanaihara *et al.*, 2005; Talbi *et al.*, 2006). Genes with high expression in the progesterone-regulated secretory phase include immune and inflammatory response, secretory proteins, extracellular matrix degradation, cholesterol transporters, signalling molecules, metabolism and detoxification (Carson *et al.*, 2002; Kao *et al.*, 2002).

Gene profiling studies have contributed significant knowledge into the biological processes that occur in the human endometrium. On a large scale, multiple cell types within the endometrium are responsible for these dynamic changes. However, little is known about the contribution made by any specific cell-type. In fact, only a few laser microdissection studies showing a small number of differentially expressed genes between endometrial epithelial and stromal cells have been undertaken (Yanaihara *et al.*, 2004; Yanaihara *et al.*, 2005; Gaide Chevronnay *et al.*, 2009). A distinct molecular signature was found between luminal and glandular epithelium using a mouse model (Niklaus & Pollard, 2006). This is a step toward understanding the cellular phenotype of human endometrial epithelium. However, the mouse and the human endometrium do not necessarily mirror one another. This is illustrated by the finding of 17βHSD-2 gene. Up-regulation of 17βHSD-2 was found in the estrous cycle of the mouse uterus (Tan *et al.*, 2003), but it was up-regulated in early secretory phase compared to proliferative phase in human (Talbi *et al.*, 2006).

Gene expression profiles of post-menopausal endometrium have also been studied (Klaassens *et al.*, 2006; Hanifi-Moghaddam *et al.*, 2007). However, these were focussed on the effects of hormone replacement therapy. Like other gene profiling studies, endometrial tissues containing multiple cell types were used, thus biological processes of specific cell types were not determined.

1.3.8 Wnt signalling in the endometrium

The Wnt signalling pathway is essential for female reproductive tract development as well as regulating endometrial proliferation and differentiation during the menstrual cycle (Miller *et al.*, 1998; Parr & McMahon, 1998; Hayashi & Spencer, 2006; Hayashi *et al.*, 2011). In human endometrium, Wnt2, Wnt3, Wnt4, Wnt5a, Wnt7a and Wnt7b were expressed in both proliferative and secretory stage of the menstrual cycle (Bui *et al.*, 1997;

Tulac *et al.*, 2003). Their mRNA expression was independent of hormonal regulation (Bui *et al.*, 1997). In adult mouse uterus, Wnt7a is localised exclusively to the luminal but not glandular epithelium and is involved in maintaining uterine pattering during development (Miller *et al.*, 1998; Miller & Sassoon, 1998). Luminal epithelial location of Wnt7a was also confirmed for human endometrium (Tulac *et al.*, 2003). Other members of the Wnt pathways including Wnt receptors and co-receptors (Fzd6, LRP6) and downstream effectors (DVL-1, GSK-3β and β-catenin) were also expressed throughout the menstrual cycle (Tulac *et al.*, 2003). Taken together the evidence suggests that the Wnt signalling pathway is involved in endometrial growth and differentiation in the normal menstrual cycle and that Wnt genes are likely regulated by steroid hormones (Nei *et al.*, 1999; Tulac *et al.*, 2003; Hou *et al.*, 2004; Tulac *et al.*, 2006; Wang *et al.*, 2009; Wang *et al.*, 2010). However, nothing is known about the Wnt pathway in basalis epithelium or in post-menopausal endometrial epithelial stem/progenitor cells.

1.3.9 Endometrial epithelial stem/progenitor cells

The human endometrium is a dynamic tissue with remarkable regenerative capacity each menstrual cycle (McLennan & Rydell, 1965; Gargett, 2007). This process of proliferation, differentiation and shedding is repeated every 28 days from puberty to menopause for at least 400 cycles during a these reproductive years (Jabbour *et al.*, 2006). Thus, the cyclical cellular turn over of human endometrium is similar in magnitude to that of the haematopoietic system, intestine and skin in which adult stem cell populations have been identified. While the concept of stem/progenitor cells residing in the basalis mediating endometrial regeneration was proposed many years ago (Fig. 1.8) (Prianishnikov, 1978; Padykula, 1991; Gargett, 2007), it was only recently that a small population of

stem/progenitor cells have been identified in human and mouse endometrium (Chan *et al.*, 2004; Chan & Gargett, 2006; Gargett & Masuda, 2010).

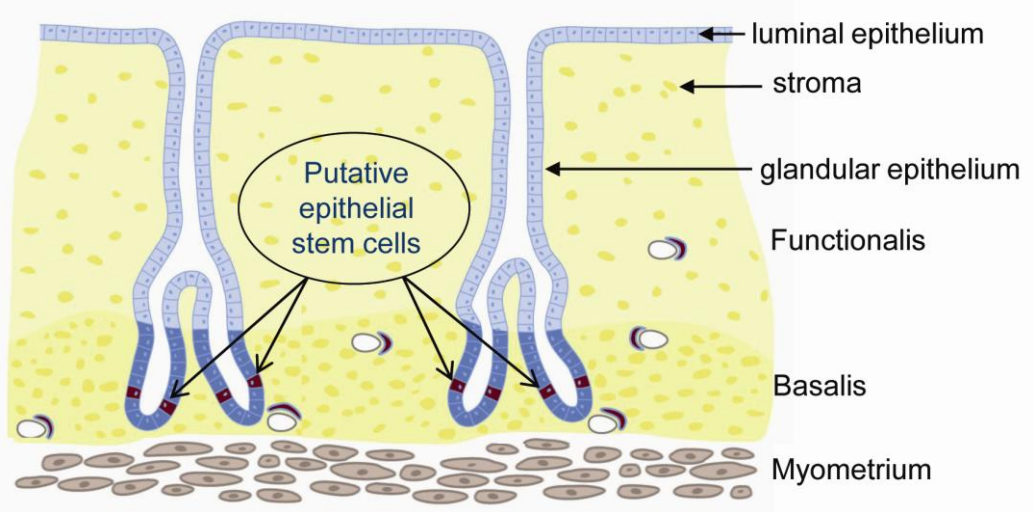


Figure 1.8 Schematic diagram showing the possible location of human endometrial epithelial stem/progenitor cells (ESP). Putative ESP cells are postulated to reside in the base of the glands in the basalis layer of the endometrium (black arrows). Adapted from Gargett, C.E (2007) *Human Reproduction Update* 13 (1): 87-101 with permission from Oxford University Press.

1.3.9.1 Evidence of stem/progenitor cells in human endometrium

1.3.9.1.1 Proliferation studies

Different proliferation rates exist between different layers in the endometrium showing low proliferation in the basalis, indicative of slow cycling, quiescent stem/progenitor cells. In contrast, high proliferation rates occur in the functionalis reflecting the more mature differentiated transit-amplifying cells undergoing cell division in response to sex steroid hormones (Ferenczy *et al.*, 1979; Conti *et al.*, 1984; Padykula *et al.*, 1989; Okulicz *et al.*, 1997). A recent report, using a more stringent proliferation marker, phosphorylated histone H3 demonstrated proliferation differences between functionalis and basalis layer, higher

rates in the functionalis compared to the basalis and apparently regulated by progesterone (Padykula, 1991; Brenner *et al.*, 2003).

1.3.9.1.2 Methylation patterns and monoclonality of endometrial glands

Endometrial epithelial stem cell proliferation was determined by counting the epigenetic errors encoded in methylation patterns in certain genes in endometrial glands (Kim *et al.*, 2005). A mathematical modelling in this study demonstrated that an individual gland contains a stem cell niche with multiple long-lived stem cells rather than a single stem cell (Kim *et al.*, 2005). In addition, gland diversity remains after menopause, indicating that a reservoir of stem cells also exist in atrophic endometrium (Kim *et al.*, 2005; Gargett, 2007).

Another study demonstrated that endometrial glands are monoclonal in origin. Clonality of individual endometrial epithelial glands were investigated using PCR amplification of non-random X chromosome inactivation of the androgen receptor and found that glands within 1-2mm proximity shared clonality (Tanaka *et al.*, 2003). This data suggests that single or multiple stem cells with uniform clonality exit at the bottom of each endometrial gland (Tanaka *et al.*, 2003; Gargett, 2007).

1.3.9.2 Epithelial stem/progenitor cells in human endometrium

Cell cloning studies of purified endometrial epithelial cells obtained from hysterectomy tissue provided the first evidence for the existence of epithelial stem/progenitor cells (Chan *et al.*, 2004). This rare clonogenic population (0.2%) of epithelial cells isolated from hysterectomy tissue comprised two distinct epithelial colony types. The large colonies (0.09%) contain large number of small, densely packed cells with high nuclear: cytoplasmic ratio, suggesting they were initiated by ESP cells. The small colonies

comprised large, loosely packed cells with low nuclear: cytoplasmic ratio, a characteristic of more differentiated TA cells (Chan *et al.*, 2004). Further work has shown that the percentage of clonogenic cells remained unchanged across the menstrual cycle (Schwab *et al.*, 2005). There was no difference between active, cycling and inactive, non-cycling endometrium including post-menopausal endometrium (Schwab *et al.*, 2005). This finding suggests that putative stem/progenitor cells are responsible for endometrial regeneration and their frequency is independent of hormone regulation (Schwab *et al.*, 2005; Teixeira *et al.*, 2008). This concept is strengthened by the ability of atrophic post-menopausal endometrium to regenerate when women are given hormone therapy (Feeley & Wells, 2001; Gargett, 2007).

Subsequent studies indicated that large epithelial colony-forming activity (CFU) had substantial self-renewal capacity, undergoing serial cloning 2.5 times when seeded at extremely low seeding densities. In contrast, cells initiating small colonies underwent self-renewal 0.5 times and had much lower proliferative potential (Gargett *et al.*, 2009; Gargett & Masuda, 2010). The large epithelial CFU also exhibited high proliferative potential, undergoing 34 population doublings. Additionally, the large epithelial CFU differentiated into large cytokeratin expressing gland-like structures (Gargett *et al.*, 2009). Taken together, this evidence indicates that large CFU initiating the colonies are likely epithelial stem/progenitor cells, which likely reside in the basalis. Small CFU may be initiated by the transit-amplifying cells likely resident in the functionalis and contributing to extensive proliferation during the proliferative phase of the menstrual cycle (Gargett *et al.*, 2009; Gargett & Masuda, 2010).

1.3.9.3 Side population and tissue reconstitution in human endometrium

Endometrial stem cells have been identified as side population (SP) cells. Endometrial SP cell population are enriched in $CD9^{-}CD13^{-}$ fraction (CD9, epithelial and CD13, stromal markers respectively) (Kato *et al.*, 2007). These SP cells survived in long-term cultures producing CD9-expressing glands and CD13-expressing stroma-like cells (Kato *et al.*, 2007). Endometrial SP cells also reconstituted endometrial tissue *in vivo* and expressed markers of different cell types including epithelial, stromal and endothelial cells (Cervelló *et al.*, 2010; Masuda *et al.*, 2010). SP cell sorted from cultured endometrial cells were primarily in G1 of the cell cycle (Tsuji *et al.*, 2008; Gargett & Masuda, 2010). SP cells derived from primary endometrial cell lines also demonstrated the ability to reconstitute endometrial tissue (Cervelló *et al.*, 2011). Together, these lines of evidence suggest that endometrial SP cells are heterogeneous and contain several different sub-populations capable of producing endometrial-like tissue *in vitro* and *in vivo*. However, it is not clear from these studies if there is a distinct ESP cells or stem/progenitor cell population in the SP fraction.

Unfractionated endometrial epithelial and stromal cell suspensions have the capacity to reconstitute endometrial-like tissue when transplanted under the kidney capsule of ovariectomised, estrogen-supplemented NOD/SCID/γ_c^{null} (NOG) mice (Masuda *et al.*, 2007). This tissue reconstitution recapitulated a well-organised functional endometrium adjacent to the myometrial layer, expressing epithelial (cytokeratin and CD9) and stromal (CD10 and CD13) markers which was responsive to estrogen and progesterone hormones (Masuda *et al.*, 2007; Gargett & Masuda, 2010). This study supports the existence of endometrial stem/progenitor cells in the human endometrium but it does not allow the prospective isolating of ESP cells from the human endometrium.

1.3.9.4 Epithelial stem/progenitor cells in mouse endometrium

A small population of epithelial stem/progenitor cells with stem-like properties were identified in the mouse endometrium using a label-retaining cell (LRC) technique (Chan & Gargett, 2006; Cervelló *et al.*, 2007). Slow-cycling, quiescent stem/progenitor cells retaining the BrdU label are known as LRC. When postnatal pups were labelled and chased for 4 – 8 weeks, 3% of mouse endometrial epithelial cells were identified as $BrdU^+$ LRC. The epithelial LRC were located primarily in the luminal epithelium and did not express ERα. Interestingly, these epithelial LRC were the first cells to proliferate in response to estrogen stimulation in prepubertal mice, suggesting that estrogen drives the endometrial epithelial regeneration indirectly via ERα-expressing niche cells which recruit epithelial LRC into the cell cycle (Chan & Gargett, 2006; Chan *et al.*, 2011).

In a different mouse model examining endometrial breakdown and repair, epithelial LRC were found in the glands located in the basal regions adjacent to the myometrium (Kaitu'u-Lino *et al.*, 2010). This study showed that glandular epithelial LRC were ERα negative and may be involved in the repair of luminal epithelium. The finding suggests that glandular epithelium LRC could be the candidate epithelial stem/progenitor cells inducing epithelial repair after tissue damage in the absence of hormonal support (Kaitu'u-Lino *et al.*, 2007; Kaitu'u-Lino *et al.*, 2010).

At present, the two LRC models of mouse endometrial regeneration and repair provides evidence that luminal LRC may be responsible for luminal epithelium regeneration during development while glandular LRC could have key role in replenishing the shed and lost luminal epithelium in adult cycling endometrium. The repair model appears to recapitulate postmenstrual re-epithelialisation of normal human endometrium (Gargett & Masuda, 2010). The absence of ERα in luminal and glandular epithelial LRC suggests that

endometrial LRC require paracrine signals from adjacent niche cells to activate these potential ESP cells to enter the cell cycle and initiate epithelial regeneration (Chan *et al.*, 2011). It also suggests that there are two populations of ESP cells that may exist in the endometrium to maintain epithelial cell homeostasis.

1.3.10 Sources of endometrial stem/progenitor cells

Endometrial stem/progenitor cells may originate from fetal stem cells remaining in adult tissue (Gargett, 2007). However, there is evidence suggesting that bone marrow may also be a source of endometrial stem/progenitor cells. A previous study has provided evidence that bone marrow cells could be responsible for endometrial regeneration (Taylor, 2004). In this study, donor-derived endometrial cells were detected in endometrial biopsies from women who previously received a single HLA-antigen mismatched bone marrow transplant (Taylor, 2004). In several subsequent investigations, rare human male donor-derived bone marrow cells were also demonstrated in endometrial glands and stroma in female recipients (Ikoma *et al.*, 2009). In mouse studies, using a gender mismatch bone marrow transplant model, rare male cells were located in the uterine endometrium of female mice (Du & Taylor, 2007). More specifically, circulating genetically labelled $CD45^{+}$ bone marrow cells gave rise to a small number of uterine epithelial cells in mice (Bratincsak *et al.*, 2007). A recent study has demonstrated that bone marrow-derived mesenchymal stem cells have the ability to differentiate into endometrial stromal-like cells upon protein kinase A pathway activation (Aghajanova *et al.*, 2010). These cells exhibited decidual cell morphology and expressed markers of decidualisation. This body of evidence indicates that non-uterine cells could be the source of some endometrial stem/progenitor cells responsible for endometrial regeneration, however the contribution appears small.

Further research is required to investigate whether bone marrow-derived cells differentiate and can produce endometrial tissue with two distinct layers.

1.3.11 Markers of endometrial stem/progenitor cells

Compelling evidence supports the existence of endometrial epithelial stem/progenitor cells, however, the lack of available markers prevents their prospective isolation and their location is currently unknown. At the commencement of this study, there were no known markers of endometrial ESP cells. However, recent evidence demonstrated that a large proportion of endometrial glands expressed Musashi-1, an intestinal epithelial stem cell marker (Götte *et al.*, 2008). Musashi-1^{+} epithelial cells were also found in higher numbers in the basalis of proliferative phase compared to the functionalis and were more frequent than stromal Musashi-1 expressing cells (Götte *et al.*, 2008). While this study indicates the possible location of endometrial epithelial stem/progenitor cells, no functional studies such as clonogenic activity and tissue reconstitution on Musashi-1 endometrial epithelial cells have been undertaken. Therefore, no conclusions can be drawn on whether Musashi-1 is a marker of functional endometrial ESP cells.

While there are no known markers of endometrial ESP cells, co-expression of two perivascular markers, CD146 and PDGF-Rβ markers have identified human endometrial stromal/mesenchymal stem cells. $CD146^{+}PDGF\text{-}R\beta^{+}$ cells enriched for rare endometrial mesenchymal stem cells (MSC) with high clonogenic activity compared to unfractionated stromal cells and $CD146^{-}PDGF\text{-}R\beta^{-}$ populations (Schwab & Gargett, 2007). $CD146^{+}PDGF\text{-}R\beta^{+}$ cells also expressed mesenchymal stem cell phenotypic markers and underwent multi-lineage differentiation into adipogenic, osteogenic, myogenic and chondrogenic cells (Schwab & Gargett, 2007). Recently, work from the same laboratory demonstrated that W5C5 marker can isolate endometrial MSC expressing similar

mesenchymal markers and underwent multi-lineage differentiation (Masuda *et al.*, 2011). These markers showed that endometrial MSC are present in both basalis and functionalis. Taken together, it is imperative that candidate markers of endometrial epithelial stem/progenitor cells are identified to enable their prospective isolation. Only when makers of endometrial ESP cells are identified can their molecular and cellular properties be examined and their location determined. This will then allow studies examining the role of endometrial epithelial stem/progenitor cells in gynaecological disorders such as endometrial cancer and endometriosis.

1.3.12 Technical challenges in culturing endometrial epithelial cells

Flow cytometry sorting has been adopted to isolate and sort endometrial stromal / mesenchymal stem cell population, enabling their MSC properties to be investigated in clonal cultures and their differentiation into multi-lineages (Schwab & Gargett, 2007; Schwab et al., 2008). This technique has not been use to isolate endometrial epithelial cells. This is because the endometrial ESP cells have not been identified due to lack of specific markers for their identification, and the inability to maintain long term cultures of endometrial epithelial cells. For a long time, many attempts have been made to develop a successful culturing system for human endometrial epithelial cells, using techniques including enzyme digestion and differential filtration. However, it was found difficult, and epithelial cell growth greatly depended on their survival after the initial dissociation (Chaminadas et al., 1986; Fernandez-Shaw et al., 1992). Stromal feeders were required to improve their clonogenic activity in serum-free medium (Chan et al., 2004). Taken together the evidence indicates that an optimal isolation and culturing condition is very important for identifying and characterising endometrial ESP cells.

1.4 Endometrial cancer

1.4.1 Incidence, symptoms and risk factors

Endometrial carcinoma (EnCa) is the seventh most common cancer worldwide (Amant *et al.*, 2005) and is the most common gynaecological malignancy of the female reproductive tract (Di Cristofano & Ellenson, 2007). In the year 2005, and 2006, EnCa was ranked the 6th and 4th most common cancer in American and Australian women respectively (AACR, 2008; Edwards *et al.*, 2010). In Australia, EnCa accounts for 4.2% of total cancer cases and in 2007, 338 deaths were reported from the 1, 942 female individuals diagnosed with endometrial cancer. The incidence rates have increased in the last decade due to the obesity epidemic and aging population (AACR, 2008). Similarly, data from the American Cancer Society estimated 43,470 new cases in 2010 and 7,950 deaths (Jemal *et al.*, 2010). Although, the incidence of EnCa is higher in women of North America and Europe, the risk and mortality rates are higher in women of less-developed countries or low socioeconomic status (Hill *et al.*, 1995; Madison *et al.*, 2004; Amant *et al.*, 2005). Nevertheless, the 5 year survival rate is about 80% (Bokhman, 1983).

Nearly 90% of endometrial cases present with abnormal uterine bleeding and diagnosis is usually made at an early stage of the condition, with age ranging between 50 – 60 years of age and explaining the 5 year survival rate (Sorosky, 2008). Obesity, smoking, nulliparity, polycystic ovary syndrome and high body mass index increase the risk of endometrial cancer (Goodman *et al.*, 1997; Hinkula *et al.*, 2002; Calle *et al.*, 2003; Schouten *et al.*, 2004). Additionally, post-menopausal women with a uterus who are on estrogen only hormone replacement therapy have an increased risk of developing endometrial cancer (Parslov *et al.*, 2000; Lacey *et al.*, 2005; Lacey *et al.*, 2007).

Diagnosis is often carried out by obtaining endometrial tissue biopsy and studying glandular and stromal histological features. Post-menopausal bleeding increases the risk of EnCa, thus biopsy is recommended for women over 40 years of age presenting with abnormal uterine bleeding (Gredmark *et al.*, 1995; Amant *et al.*, 2005). Total hysterectomy is the primary treatment recommended for women with EnCa (Sorosky, 2008).

1.4.2 Types, stage and histological features

In 1983, a large study suggested that endometrial cancer could be classified into two types based on aetiology and clinical features (Bokhman, 1983). Type I carcinoma is also known as endometrioid carcinoma, is more common, occuring in 70-80% of cases. Type I EnCa is defined as estrogen dependent, of low grade with endometrioid differentiation. It has a good prognosis, arises from hyperplastic endometrium and tends to occur in young peri-menopausal women (Table 1.2) (Bokhman, 1983; Amant *et al.*, 2005; Di Cristofano & Ellenson, 2007). Type I EnCa is preceded by endometrial hyperplasia, which manifest as an increase in normal endometrial epithelial proliferation, possibly due to high levels of unopposed estrogen (Di Cristofano & Ellenson, 2007; Mutter *et al.*, 2007). Retrospective studies have confirmed that women who were previously diagnosed with atypical endometrial hyperplasia had a higher incidence of endometrioid carcinoma (Trimble *et al.*, 2006; Pennant *et al.*, 2008; Hahn *et al.*, 2010). Type I EnCa is often classified into 3 grades; where grade 1 is well differentiated, grade 2 is moderately differentiated and grade 3 is poorly differentiated (Table 1.3) (Sorosky, 2008). The main features of type I EnCa are squamous, mucinous metaplasia with smooth luminal contours (Soslow *et al.*, 2009) (Fig. 1.9A-C).

In contrast, type II EnCa classified as non-endometrioid carcinoma. It has histological features of serous papillary or clear cell carcinoma, resembling fallopian tube-like

morphology (Fig. 1.9D-E). Type II EnCa is estrogen independent arises from atrophic endometrium in older post-menopausal women (Table 1.2). It is a high grade cancer and women frequently undergo relapse and metastasis (Bokhman, 1983; Di Cristofano & Ellenson, 2007; Sorosky, 2008).

TABLE 1.2 Classifications of type I and II endometrial cancer

Type I	**Type II**
Endometrioid adenocarcinoma	Non-endometrioid carcinoma
Associated with endometrial hyperplasia	Associated with EIC, serous or clear cells
Estrogen dependent	Estrogen independent
Low grade	High grade
Peri-menopausal women	Post-menopausal women
Good prognosis	Poor prognosis
Minimal myometrial invasion	Deep myometrial invasion
Main genetic mutations: PTEN, K-ras, microsatellite instability, β-catenin	Main genetic mutations: p53, Her-2

EIC, endometrial intraepithelial carcinoma. This table summarises information from multiple sources (Amant *et al.*, 2005; Di Cristofano & Ellenson, 2007; Soslow *et al.*, 2009).

Serous carcinoma is the most aggressive form of type II EnCa and is characterised by complex pattern of papillary features with highly atypical cells and non-squamous, morular and jagged luminal surfaces (Silverberg *et al.*, 2003; Soslow *et al.*, 2009) (Fig. 1.9D). Serous carcinoma often results from the precursor lesion, endometrial intraepithelial carcinoma (EIC) (Amant *et al.*, 2005; Di Cristofano & Ellenson, 2007). Histologically, EIC is diagnosed when surface epithelium is replaced by pleimorphic tumour cells without stromal invasion (Amant *et al.*, 2005). Clear cell carcinoma is a rare form of type II EnCa accounting for just 3–6%. Clear cell carcinoma is seen in post-menopausal women with an average mean age of 68 years. Features of clear cell carcinoma include clear or hobnail cells with cytoplasmic clearing owing to its glycogen contents and hyaline stroma (Hurteau *et al.*, 2008; Soslow *et al.*, 2009) (Fig. 1.9E).

Malignant Müllerian mixed tumour (MMMT) also known as carcinosarcoma, is another form of endometrial cancer that comprises an admixture of epithelial and stromal elements of carcinoma and sarcoma cell types and are classified as either type I or type II depending on the histogenetical features of the carcinomatous component (Silverberg *et al.*, 2003; Lax, 2004) (Fig. 1.9F). MMMT generally occurs in post-menopausal women (Silverberg *et al.*, 2003; Soslow *et al.*, 2009). Since there are two distinct cancer cell types exist within MMMT, it is appeared to be the best model to study the genetic relationship between the types (Lax, 2004).

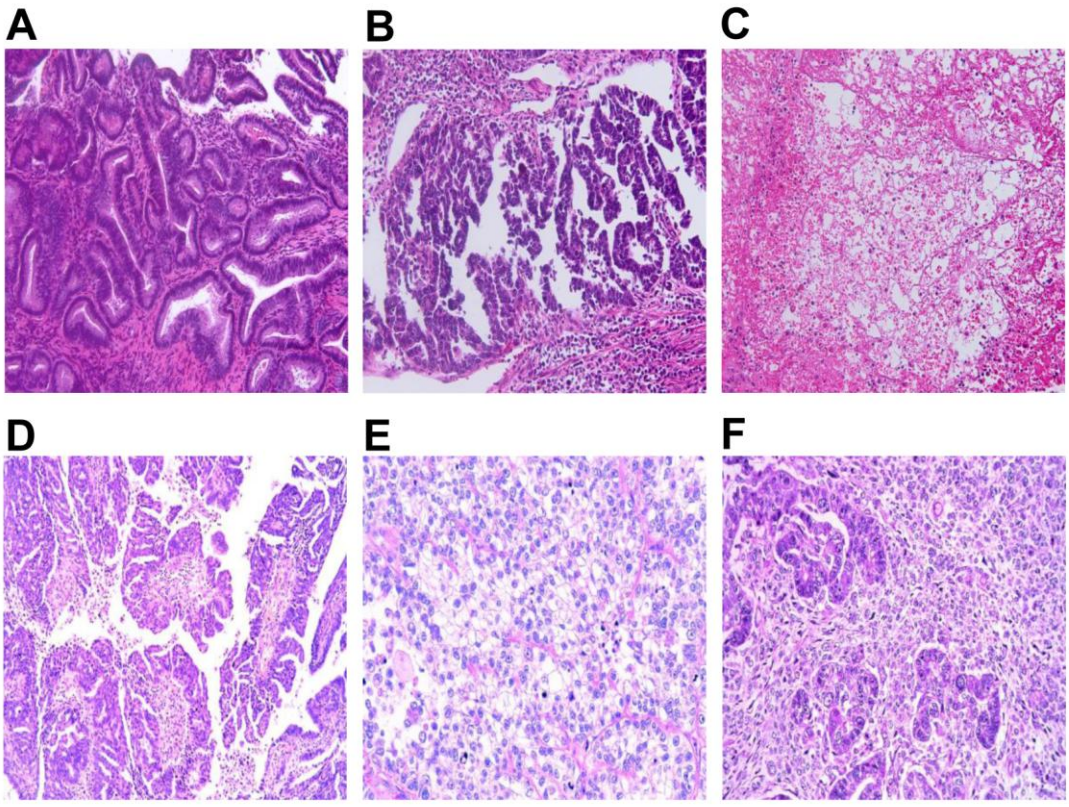


Figure 1.9 Histological grades and types of endometrial cancer. There are two types of endometrial cancer. Type I EnCa are divided into **A)** grade 1. **B)** grade 2 and **C)** grade 3. Type II EnCa consists of **D)** Serous papillary carcinoma and **E)** Clear cell carcinoma. **F)** Malignant Müllerian mixed tumour (MMMT) showing both epithelial and mesenchymal features. Images for D-F were adapted from *WHO classification of tumours* online book (Reference as Silverberg et al 2003).

According to the International Federation of Gynaecology and Obstetrics (FIGO), surgical staging of EnCa is essential to determine the prognosis of the disease. Currently there are 4 stages of disease progression. Specific description of the different stages is outlined in Table 1.3 (Amant *et al.*, 2005; Di Cristofano & Ellenson, 2007; Creasman, 2009).

TABLE 1.3 Classification of endometrial cancer type I and II sub-types and FIGO surgical staging use to predict cancer prognosis

Endometrial cancer classifications	Description
Type I endometrioid	
Grade 1	well-differentiated, ≤ 5% non-squamous solid growth pattern
Grade 2	moderately differentiated, 6- 50% of non-squamous growth pattern
Grade 3	poorly-differentiated, > 50% of non-squamous growth area
Type II non-endometrioid	
Serous carcinoma	complex pattern of papillary features with highly atypical cells
Clear cell carcinoma	clear or hobnail cells with clear cytoplasm
Malignant Müllerian mixed tumour (MMMT)	a mixture of epithelial and mesenchymal malignant components, high grade pleomorphic nuclei, papillary,
Staging of endometrial cancer	
I	tumour confined to uterine corpus
IA	tumour showed invasion of < 50% of the myometrium
IB	tumour showed invasion of one-half or more of the myometrium
II	tumours invades the cervix
III	tumour involves the vagina, peritoneal cavity lymph nodes in close proximity
IV	tumour spreads to the bladder, rectum and abdominal areas

This table summarises information of multiple sources (Silverberg *et al.*, 2003; Amant *et al.*, 2005; Creasman *et al.*, 2006; Di Cristofano & Ellenson, 2007; Creasman, 2009; Sobin *et al.*, 2009).

1.4.3 Genetic alterations in endometrial cancer

The most common mutations associated with type I endometrioid carcinoma are in the PTEN and β-catenin and K-ras genes and microsatellite instability (MSI) (Table 1.4) (Di Cristofano & Ellenson, 2007). PTEN mutations (tumour suppressor gene) are frequently found in type I endometrioid cancer (83%) and in about 20% of endometrial hyperplasia (Mutter *et al.*, 2000). Mutations of the PTEN genes are also associated with microsatellite instability (a common molecular alteration in EnCa) (Mutter *et al.*, 2000). Together, this

indicates that PTEN and MSI mutations are possibly involve in the early development of EnCa (Levine *et al.*, 1998; Maxwell *et al.*, 1998).

Mutations of K-ras (oncogene), specifically codon 12 was found in 10 - 30% of type I EnCa. The frequency of K-ras mutation is often higher in grade 2 compared to grade 1 or 3 endometrioid tumours (Lax *et al.*, 2000) and it is also commonly found with MSI (Prat *et al.*, 2007). However, there is no direct correlation between K-ras mutation with the age-onset or histological grading of the disease (Esteller *et al.*, 1997; Lax *et al.*, 2000; Di Cristofano & Ellenson, 2007).

TABLE 1.4 Molecular genetics of endometrial cancer

Type of gene mutations	Frequency of mutation
PTEN	30 - 80% endometrioid, 20% of endometrial hyperplasia
K-ras	10 - 30% endometrioid
Microstatellite instability (MSI)	20% of sporadic endometrioid
β-catenin	15 - 20% endometrioid
p53	90% of serous, 75% of EIC, 10 - 20% of grade 3 endometrioid
Her-2	18 - 80% serous and clear cell

This table summarises information of multiple sources (Amant *et al.*, 2005; Di Cristofano & Ellenson, 2007; Sorosky, 2008; Creasman, 2009).

β-catenin (*CTNNB1* gene) mutation is a another molecular feature of 15 - 20% type I EnCa (Scholten *et al.*, 2003; Di Cristofano & Ellenson, 2007). Studies suggest that nuclear accumulation of β-catenin occurs in 38% of cases and this is due to the disruption in the Wnt signalling pathway (Fukuchi *et al.*, 1998; Machin *et al.*, 2002; Moreno-Bueno *et al.*, 2002; Di Cristofano & Ellenson, 2007). The Wnt signalling pathway is important in the development of the female reproductive tract and is regulated during the menstrual cycle under the influence of ovarian hormones (Miller *et al.*, 1998; Tulac *et al.*, 2003; Wang *et al.*, 2009). Thus it is possible that alterations in the Wnt signalling pathway likely

contribute to endometrial cancer development (Di Cristofano & Ellenson, 2007; Wang *et al.*, 2009; Wang *et al.*, 2010).

P53 mutations occur primarily in type II non-endometrioid (or serous) carcinoma and accounts for 90% of serous cases and 75% of the precursor EIC. Several studies have reported that mutations in the p53 gene contributed to 10 - 20% of grade 3 endometrioid carcinoma (Enomoto *et al.*, 1993; Di Cristofano & Ellenson, 2007). This is consistent with previous sequence analysis of p53 mutations which were absent in grade 1, rare in grade 2 and more common in grade 3 (Lax *et al.*, 2000). Recent studies confirm that the p53 signature is a feature of endometrial serous carcinoma (Jarboe *et al.*, 2009).

Her-2 (also known as human epidermal growth factor type II receptor, EGFR2) is another oncogene overexpressed in 18 - 80% of type II EnCa. It is associated with poor prognosis, resistance to chemotherapy, metastasis and recurrence (Khalifa *et al.*, 1994). Overexpression of Her2 is often related to inactivation of p16, which causes alterations in signal transduction pathways including MAPK and PI3K, resulting in increasing cell proliferation (Morrison *et al.*, 2006; Konecny *et al.*, 2008; Bansal *et al.*, 2009).

1.4.4 Hormones and hormone receptors in endometrial cancer

In the normal endometrium, estrogen and progesterone are essential for regulating cellular proliferation and differentiation respectively and this signal is mediated via interaction with their receptors ERα, ERβ, PR-A and PR-B (Jabbour *et al.*, 2006). Because type I EnCa is associated with unopposed estrogen stimulation, studies have examined hormone receptors in the hope of finding a correlation between hormone levels, hormone receptor expression and EnCa prognosis and staging. Estrogen receptors are expressed by the majority of EnCa tissues (Satyaswaroop & Mortel, 1993; Ito *et al.*, 2007), and ERα

expression is more common in well-differentiated EnCa compared to ERβ (Utsunomiya & Suzuki T, 2000; Mylonas *et al.*, 2009). ERα is the predominant receptor known to mediate estrogen effects in EnCa (Utsunomiya & Suzuki T, 2000; Ito *et al.*, 2007). ERα levels decreased with clinical stage, grade and myometrial invasion (Fujimoto & Sato, 2009), while high levels of ERβ were found in EnCa with severe myometrial invasion (Takama *et al.*, 2001).

The loss of PR expression is likely associated with poor prognosis of EnCa (Jeon *et al.*, 2006; Shabani *et al.*, 2007). Overexpression of PR-A relates to increased epithelial cell mass (Takama *et al.*, 2001; Shabani *et al.*, 2007). In well-differentiated EnCa cell lines, Ishikawa, PR-B was highly expressed, especially on the addition of estrogen (Kumar *et al.*, 1998), while in poorly-differentiated EnCa cell line (Hec50), it was shown that progesterone acts via PR-B to inhibit endometrial cancer cell invasion slow (Dai *et al.*, 2002).

1.4.5 Endometrial cancer stem cells

A recent study from our laboratory has shown evidence for endometrial cancer stem cells (Hubbard *et al.*, 2009). A small population (< 1%) of freshly isolated endometrial cancer cells demonstrated clonogenic activity and self-renewal capacity *in vitro* and *in vivo* in both types and grade of endometrial cancer. Clonally-derived endometrial cancer cells expressed several stem cell self-renewal genes including *BMI-1*, *NANOG* and *SOX2*. Isolated endometrial cancer cells transplanted in limiting dilution into immunocompromised mice initiated tumours, recapitulating the parent tumour histoarchitecture and marker expression of cytokeratin, vimentin, ERα and PR (Hubbard *et al.*, 2009). These tumours could be serially transplanted up to five times and required fewer

cells to establish tumours with increasing passage (Hubbard *et al.*, 2009; Hubbard & Gargett, 2010).

Side population (SP) cells have been detected in endometrial cancer and endometrial cancer cell lines (AN3CA, HEC1-A, HEC-1B, Ishikawa and SKUT-2) (Friel *et al.*, 2008; Kato *et al.*, 2010). AN3CA side population cells exhibited stem cell properties of low proliferation, self-renewal and chemoresistance. This rare SP population proliferated slower compared to the non-SP cell fraction and were mostly concentrated in G1 phase of the cell cycle. The percentage of SP cells remained unchanged when passaged multiple times, indicating a quiescent cell population responsible for tumour repopulation *in vivo*. Additionally, AN3CA side population cells were enriched for tumour-initiating cells when examined *in vivo* (Friel *et al.*, 2008; Teixeira *et al.*, 2008). Similarly, Hec-1 side population cells were clonogenic, underwent self-renewal and initiated large tumours compared to non-SP cells when transplanted into mice (Götte, 2010; Kato *et al.*, 2010).

These findings collectively provide substantial evidence that cancer stem cells are present in endometrial cancer and established endometrial cancer cell lines. This is in line with a previous study which demonstrated that uterine carcinosarcoma was derived from a single stem cell (Gorai *et al.*, 1997).

1.4.6 Markers of endometrial cancer stem cells

The intestinal epithelial stem cell marker, Musashi-1 is expressed by both normal endometrial cells and endometrial cancer cells suggestive that it may be a marker of cancer stem cells in endometrial cancer (Götte *et al.*, 2008). CD133 is another marker which has been claimed to isolate endometrial cancer stem cells (Rutella *et al.*, 2009). $CD133^{+}$ cells had high clonogenic activity, chemoresistance and higher proliferative rate, particularly in

the presence of estrogen compared to $CD133^-$ cells. However, both $CD133^+$ and $CD133^-$ populations were incapable of forming tumour when transplanted into mice (Rutella *et al.*, 2009). Recent work from our laboratory provided *in vitro* and *in vivo* evidence showing that CD133 populations do not enrich for endometrial cancer stem cells (Hubbard, 2010). CD133 may be enriched for endometrial cancer cells with stem cell properties but further evidence is required to determine if CD133 can isolate endometrial cancer stem cells.

These findings support the concept that rare cancer stem cells are responsible for the initiation, progression, chemoresistance and metastasis of endometrial cancer. Since the commencement of this thesis, there were no known markers that defined this population and their existence as the studies quotes above were published during my candidature. The identification of candidate surface markers is essential to enable the prospective isolation of rare endometrial cancer stem cells. Subsequently, it will help elucidate mechanism that cancer stem cells may play in endometrial cancer initiation and development.

1.5 Hypotheses

1.5.1 Overall hypothesis

Human endometrium and endometrial cancer contain a rare population of epithelial stem/progenitor cells which can be identified by surface markers.

1.5.2 Specific hypotheses

Chapter 2 A robust isolation protocol for human endometrial epithelial cells will enable their long term culture for the assessment and identification of candidate markers of endometrial epithelial stem/progenitor cells.

Chapter 3 The basalis epithelium of pre-menopausal endometrium is similar to post-menopausal endometrial epithelium, sharing a common gene signature but differential signalling pathways in comparison with functionalis epithelium.

Chapter 4 N-cadherin, a surface marker differentially expressed between pre-menopausal and post-menopausal endometrial epithelial cells isolates putative human endometrial epithelial stem/progenitor cells.

Chapter 5 A small sub-population of endometrial cancer cells express known cancer stem cells markers and exhibit stem cell properties.

1.6 Aims

1.6.1 Overall aim

To identify markers for the prospective isolation of human endometrial epithelial stem/progenitor cells and endometrial cancer stem cells.

1.6.2 Specific aims

Chapter 2 To optimise currently available methods for the isolation of human endometrial epithelial cells using enzyme digestion, flow cytometry sorting and clonal cultures.

Chapter 3 To compare the transcriptional gene profiles of purified epithelial cells isolated from full thickness pre-menopausal and basalis-like post-menopausal endometrium. To determine if the basalis of pre-menopausal and post-menopausal endometrium have similar gene profiles and to identify common signalling pathways or candidate markers.

Chapter 4 To compare the transcriptional gene profiles (from Chapter 3) of purified epithelial cells isolated from full thickness pre-menopausal and basalis-like post-menopausal endometrium. Specifically, to use these gene profiles to identify several surface maker genes overexpressed in the basalis epithelium and post-menopausal epithelium compared to premenopausal functionalis epithelium and examine their potential to isolate endometrial epithelial stem/progenitor cells.

Chapter 5 To determine if the expression of known adult stem cell markers will identify endometrial cancer stem cells with colony-forming activity and sphere formation.

Chapter 2

Isolation, culture and characterisation of human endometrial epithelial cells

Declaration

Monash University

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Responsible for refining the research question, designing and performing the experiments, collecting data for all figures except Supplemental figure 2, interpreting and preparing all figures and tables and manuscript writing	75 %

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Charmaine Tan	Optimised the stromal feeder layer conditions, assisted with experimental work, helped revise the manuscript	10 %
Jyothsna Rao	Assisted with experimental work, collected stromal-conditioned media	5 %
Caroline Gargett	Participated in the study design, helped revise the manuscript, read and approved the final manuscript	10 %

Candidate's Signature	[redacted]	Date 9 Dec 2011

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	The Ritchie Centre, Monash Institute of Medical Research Department of Obstetrics and Gynaecology, Monash University Monash Medical Centre, 246 Clayton Road, Clayton, Victoria, 3168, Australia

[Please note that the location(s) must be institutional in nature, and should be indicated here as a department, centre or institute, with specific campus identification where relevant.]

Signature 1	[redacted]	**Date** 06/12/11
Signature 2		06/12/11
Signature 3		09/12/11

Abstract

The presence of rare clonogenic cells in the human endometrium suggest the existence of adult stem/progenitor cells in the basalis that are not shed during menstruation. A robust isolation and characterisation protocol for endometrial epithelial stem/progenitor cells will enable their identification and to study their biological properties and their possible involvement in endometrial proliferative disorders. Although, several different techniques have been established for isolating and maintaining long term culture of human endometrial epithelial cells, these have been proven difficult. This study aims to describe a step-by step protocol for the successful isolation of pure endometrial epithelial single cell suspensions using combination of enzymatic digestion, differential filtration and flow cytometry sorting. Here, the effects of stromal cells, growth factors and extracellular matrices on human endometrial epithelial cell attachment, growth and survival are also examined. This chapter is formatted as a manuscript suitable for submission to a Method Protocol journal.

2.1 Introduction

Similar to other regenerative tissues including the haematopoietic system, intestine and skin, the human endometrium undergoes extensive proliferation and monthly remodelling. This cyclical process of cell proliferation, differentiation and shedding is driven by fluctuating levels of circulating estrogen and progesterone (McLennan & Rydell, 1965; Jabbour *et al.*, 2006). The endometrium comprises two layers, the upper functionalis layer which is shed during menstruation, while the lower basalis layer remains, and is thought to be the site harbouring resident stem/progenitor cells responsible for its remarkable regeneration (Padykula *et al.*, 1989; Padykula, 1991; Gargett, 2007). This is in line with

evidence for the existence of rare clonogenic and side population cells in the endometrium (Chan *et al.*, 2004; Schwab *et al.*, 2005; Kato *et al.*, 2007; Cervelló *et al.*, 2010; Masuda *et al.*, 2010) and that several growth factors including epidermal growth factor (EGF) are essential for clonogenic activity (Chan *et al.*, 2004; Gargett, 2006).

Each month, at least 4 - 7 mm of mucosal growth occurs in the endometrium and this requires the orchestration of hormone signals and cell-cell interactions (Jabbour *et al.*, 2006; Gargett, 2007). Physical and paracrine interactions between epithelial and stromal cells are important and factors secreted by stromal cells are essential for epithelial cell proliferation (Cunha & Lung, 1979; Roberts *et al.*, 1988; Kurita *et al.*, 2001). For example, estrogen-stimulated epithelial proliferation is mediated via estrogen receptors on stromal cells, which may release epidermal growth factor which is essential for endometrial epithelial cell clonogenicity (Cooke *et al.*, 1997; Chan *et al.*, 2004; Gargett, 2006, 2007).

For more than 30 years, attempts have been made to develop different approaches to isolate human endometrial cells and to characterise the composing cell types. These methods include the use of collagenase digestion and differential filtration based on the size of the sieve (Kirk *et al.*, 1978; Satyaswaroop *et al.*, 1979). However, these reports have indicated that it was difficult to achieve pure cell populations and to maintain long term culture. In these studies, tissues were obtained from endometrial biopsy and curettages rather than full thickness endometrium (Kirk *et al.*, 1978; Viganò *et al.*, 1993). Endometrial biopsy and curettages usually contain cells primarily from the functionalis while full thickness tissue from hysterectomy comprises both functionalis and basalis. Epithelial cells in the functionalis are mature, differentiated cells of the secretory glands that will be shed during menstruation whereas cells from the basalis will include potential

stem/progenitor cells responsible for the cyclical regeneration and possibly maintain long term primary cultures (Ferenczy *et al.*, 1979; Okulicz *et al.*, 1997; Gargett, 2007).

The capacity of flow cytometry sorting to separate and sort blood cell populations has been recently exploited to purify stem cells from other organ systems including epidermis, keratinocytes, prostate and endometrial stroma (Bickenbach, 2005; Li & Kaur, 2005; Schwab & Gargett, 2007; Nowak & Fuchs, 2009; Lukacs *et al.*, 2010; Masuda *et al.*, 2011). However, to our knowledge, a detailed protocol of how this technique may be applied to endometrial epithelial cell isolation has not been reported. Therefore, this study aims to provide a step-by step guide on how to successfully isolate and purify human endometrial epithelial cells as a first step in attempting to identify a rare epithelial stem/progenitor cells population. This protocol uses enzyme digestion, filtration and flow cytometry sorting. Endometrial epithelial and stromal cells selectively express EpCAM and CD90 surface markers respectively (Chan *et al.*, 2004; Schwab, 2008), thus these markers will be used in this study. Additionally, the effects of endometrial stromal cells on epithelial cell growth will also be investigated. This study will lay the groundwork for further characterisation of putative endometrial epithelial stem/progenitor cells and enable the investigation into their role in endometrial proliferative disorders.

2.2 Materials and Methods

2.2.1 Human endometrial tissue collection

Human endometrial tissues were collected from women undergoing hysterectomy for benign gynaecological conditions. Ethical approval for the study was obtained from Southern Health and Monash University Human Research and Ethics Committee and informed written consent was given by each patient for the use of their samples. Menstrual

cycle stage was assessed by an independent histopathologist according to well-established histological criteria (Noyes *et al.*, 1975). All tissues were collected in cold collection medium (DMEM-F12/HEPES, 5% new born calf serum (NCS), 1% antibiotic-antimycotic; Invitrogen, Australia) and processed within 24 hr.

2.2.2 Propagation and inactivation of endometrial stromal cells as feeder layers

Human endometrial stromal cells, isolated using the procedure below, were seeded in a T175 tissue culture flask at a density of 2,000 cells/cm^2 and grown till 70-80% confluent in stromal culture medium (sodium biocarbonate buffered DMEM-F12/, 10% fetal calf serum (FCS), 1% primocin, 1% glutamine; Invitrogen, Australia). Endometrial stromal cells were washed twice with PBS and replaced with 24 ml of warm stromal culture medium containing mitomycin-C solution (10 µg/ml; Sigma Aldrich) and incubated for 2 hr at 37ºC. Cells were washed thrice with PBS and removed from flask by incubating with Tryple Express (Invitrogen) for 4 min. The mitomycin-C inactivated stromal cells were centrifuged for 5 min at 209 g, a cell count was performed, and seeded at 3,000 cells/cm^2 into fibronectin-coated (10 µg/ml, Invitrogen) plates 4 hr prior to seeding epithelial cells. Stromal cells were also grown to make stromal conditioned medium (Supplementary Information).

2.2.3 Fibronectin-coating of culture plates

Fibronectin (BD Biosciences) was thawed slowly from -20ºC to 4ºC to room temperature (RT), vortexed and PBS added to make a stock concentration of 1 mg/ml. Culture plates were coated with sufficient volumes of fibronectin coating solution (10 µg/ml) to cover the

whole surface of the plates and left at RT in the tissue culture hood. Excess fibronectin coating solution was removed prior to seeding the cells.

2.2.4 Primary isolation of human endometrial epithelial cells to single cell suspensions

The successful isolation of pure endometrial epithelial cells will help identify endometrial epithelial stem/progenitor cells and study their unique biological properties. Previously published methods have been modified and optimised to generate this protocol which outlines the techniques used to obtain a pure and viable endometrial epithelial cell suspension, enabling their phenotypic characterisation and growth in clonal cultures.

2.2.4.1 Dissociation of unfractionated endometrial tissue

The endometrium was sliced from the adjacent myometrium layer using sterile scalpels (#22 blades) and dissecting forceps. The endometrial tissue was weighed and finely minced using scissors before being digested with 5 mg/ml collagenase type I and 40 µg/ml DNase I (Worthington Biochemical, Freehold, NJ, USA) in culture medium for 90 min at 37°C in 5% CO_2 humidified incubator using a MACSmix rotator (Miltenyi Biotec, Bergisch Gladbach, Germany). Digestion time varied between 60 - 90 min depending on the size of samples; smaller samples required shorter enzyme incubation times (see Note 1). Tissue fragments were then filtered through a sterile 40 µm cell strainer (BD Biosciences, Franklin Lakes, NJ, USA) to obtain the stromal fraction in the filtrate while leaving the glandular epithelial fraction on the strainer (Fig. 2.1A). The stromal filtrate was washed with bench medium (DMEM-F12/HEPES, 5% NCS, 1% antibiotics, Invitrogen) and centrifuged for 5 min at 209 g. The undigested epithelial glands remaining on the cell

strainer were backwashed into at sterile 50 ml tube (BD Biosciences) using culture medium and centrifuged for 5 min at 209 g (see Note 1).

2.2.4.2 Dissociation of the endometrial glandular fraction

Undigested epithelial glands were further digested with 0.8 mg/ml collagenase type II and 40 µg/ml DNase I in 5 ml culture medium for 20 min at 37°C in 5% CO_2 humidified incubator using a MACSmix rotator. The efficiency of the epithelial digestion was visualised under an inverted microscope, and when necessary, frequent pipetting using Pasteur pipette was used to obtain single cells from remaining clumps (see Note 2). The cell suspension was filtered through a sterile 40 µm cell strainer and the filtrate centrifuged for 5 min at 209 g. The epithelial single cell suspension (Fig. 2.1B) was resuspended in bench medium and a cell count using a haemocytometer was performed. Epithelial cell purity was confirmed by immunostaining with cytokeratin as described previously (Chan *et al.*, 2004) (Fig. 2.1C). The epithelial cell suspensions were then kept on ice for later use.

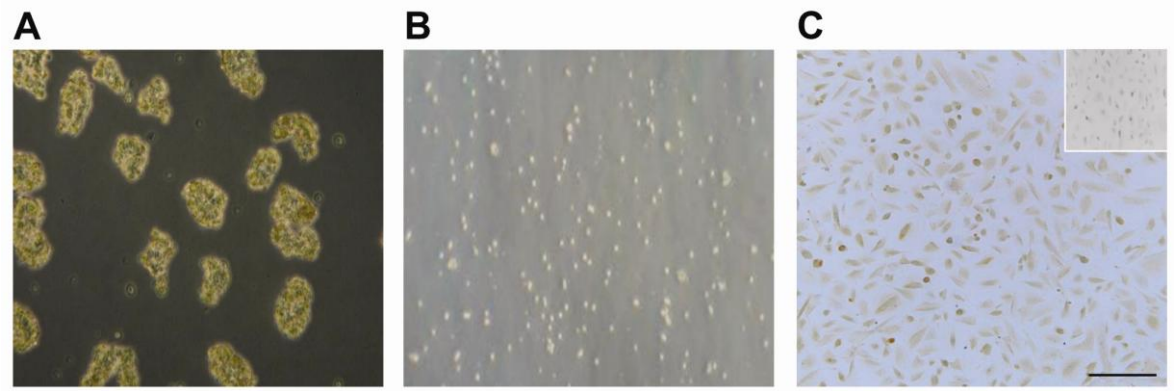


Figure 2.1 Human endometrial epithelial cell isolation. Endometrial tissue was dissociated with enzymes to obtain **A)** glandular clumps retained on the filter at first filtration step and subsequent **B)** epithelial single cell suspensions following second dissociation. **C)** Epithelial cell purity was verified by immunostaining with cytokeratin. Insets: negative control. Scale bar = 50 µm.

2.2.5 Cell staining and preparation for flow cytometric analysis and sorting

Previous studies have identified the importance of pure epithelial cell suspensions for optimal cell culture (Li *et al.*, 1998; Li & Kaur, 2005; Nowak & Fuchs, 2009; Lukacs *et al.*, 2010). Epithelial cell suspensions was sorted by flow cytometry sorting to increase the purity.

5 ml round-bottom tubes (BD Biosciences) were labelled according to Table 2.1. $1x10^5$ or > $1x10^6$ freshly isolated human endometrial epithelial cells were added to control or sample tubes, respectively, together with 2 ml of cold flow washing buffer (2% FCS/PBS), and all tubes were centrifuged for 4 min at 209 g. The supernatant was removed, taking care to ensure the cell pellet remained at the bottom of the tube. During this procedure, tubes were placed on ice inside the biohazard hood where possible. Volumes of blocking serum and primary antibody as indicated in Table 2.1 were added to the appropriate tubes, and mixed by gently flicking the tubes, before being placed onto a cold rack and covered with foil to protect from light during the first incubation for 30 min at 4ºC on a slow rotating platform. Cells were washed with 2 ml cold flow washing buffer and centrifuged as before. Volumes of blocking serum and secondary antibody as indicated in Table 2.1 were again added to the appropriate tubes, and incubated for 20 min at 4ºC on a slow rotating platform as before. Cells were washed with 2 ml cold flow washing buffer and centrifuged as before. Cells were resuspended in cold flow washing buffer containing propidium iodide (PI, 10 µg/ml, Sigma Aldrich, St Louis, MO, USA) and all tubes filtered through 30 µm, 5 ml round-bottom strainer tubes (BD Biosciences) to remove any cell clumps that could potentially clog the sorter nozzle during acquisition and sorting.

TABLE 2.1 Antibody and controls required for labelling human endometrial epithelial cells for flow cytometry analysis and sorting

Tube no.	Sample tube	Cells/ tube	BS (5 µl/ tube)	1st incubation (1° Ab, 4°C, 30min)	2nd incubation (2° Ab, 4°C, 20min)	3rd incubation
1	Unstained, cells only	$1x10^5$		--	--	--
2	Cells and PI	$1x10^5$		--	--	PI (10 µg/ml)
3	Isotype control, mouse-IgG_1	$1x10^5$	mouse	mIgG_1 (10 µg/ml)	--	
4	Anti-human CD90-APC	$1x10^5$	mouse	CD90-APC (10 µg/ml)	--	
5	Isotype control, mouse-IgG_1	$1x10^5$	rat	mIgG_1 (11.8 µg/ml)	rat anti-mouse IgG_1-PE (1 µg/ml)	--
6	Single colour control for PE, anti-human EpCAM	$1x10^5$	rat	EpCAM (11.8 µg/ml)	rat anti-mouse IgG_1-PE (1 µg/ml)	--
7	Isotype control, mouse-IgG_{2B}	$1x10^5$	goat	mIgG_{2B} (10 µg/ml)	goat anti-mouse IgG_{2B}-A488 (5 µg/ml)	--
8	Single colour control for A488, anti-human CD44	$1x10^5$	goat	CD44 (10 µg/ml)	goat anti-mouse IgG_{2B}-A488 (5 µg/ml)	--
9	Sample tube, two-colour EpCAM/CD44	$>1x10^6$	rat/ goat	EpCAM (11.8 µg/ml)/ CD44 (10 µg/ml)	rat anti-mouse IgG_1-PE (1 µg/ml)/ goat anti-mouse IgG_{2B}-A488 (5 µg/ml)/CD90-APC (10 µg/ml)	PI (10 µg/ml)

Concentrations are indicated and volume should be adjusted according to cell number. Recommended total volume is 100 µl for cell-antibody suspension per tube. BS, blocking serum; 1° Ab, primary antibody; 2° Ab, secondary antibody; PE, phycoerythrin; A488, Alexa Fluor 488; PI, propidium iodide.

2.2.6 Flow cytometry cell acquisition and sorting

The flow cytometer used in this study was a MoFlo XDP sorter (Dako Cytomation, Inc., Fort Collins, CO, USA). It requires switching on at least 2 - 3 hr prior to use for preparation to ensure accurate cell acquisition and sorting. This step varies for each instrument and should only be carried out by trained personnel, however, a general

protocol outlined below can be adopted. An appropriate nozzle (70 or 100 µm diameter) for the cell size was selected, and the instrument sterilised by flushing with sterile saline prior to cell sorting. The availability of excitation lasers for 488 nm (for measurement of forward scatter, side scatter, PE, A488 and PI) and 633 nm (for measurement of APC) was confirmed prior to the experiment (Table 2.2). Quality control of the fluorescent channels and detectors was performed by running standard fluorescent beads and setting up for single cell-passing streams. For cell sorting, a cold chamber device for cell collection was installed to ensure cell viability during long cell sorting procedure (see Note 3).

TABLE 2.2 Fluorochromes and their respective fluorescence emission wavelengths, filters and excitation laser for flow cytometry sorting

Fluorochromes	Peak emission wavelength (nm)	Band pass filters	Excitation laser wavelength (nm)
Alexa-Fluor-488 (A488)	530	530/40	Arg488
Phycoerythrin (PE)	585	570/40	Arg488
Allophycocyanin (APC)	660	670/40	HeNe633
Propidium Iodide (PI)	617	670/40	Arg488

Arg, Argon laser; HeNe, Helium-Neon laser

The MoFlo-XPD Sorter uses the Summit analysis program (V5.01, Dako) to coordinate analysis of the cell populations. Using Summit, dot plot histograms were created for forward scatter (FSC) versus side scatter (SSC) on a linear scale, and for all the relevant fluorescence channels on logarithmic scale. In order to optimise the staining results, the control tubes were analysed to enable accurate adjustments of voltage for each detector channel ensuring representative distribution of cell types within the FSC versus SSC plot (R1, Fig. 2.2A). The FSC Area versus FSC Height plot was used to create a gate that selected single cells, whilst discarding cell doublets, triplets and clumps from further

analysis (tube 1, Table 2.1) (R3, Fig. 2.2B). Dead cells and debris were electronically gated out using propidium iodide, a dye which is taken up by dead cells, using a FSC versus PI plot (tube 2, Table 2.1, R2, Fig. 2.2C).

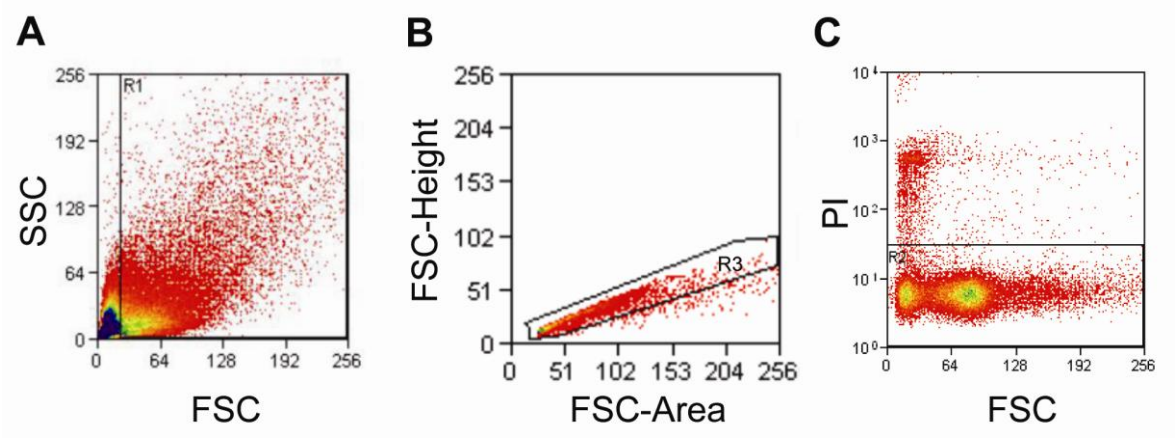


Figure 2.2 Flow cytometry profile of human endometrial epithelial single cell suspensions. **(A)** Forward scatter (FSC) vs Side scatter (SSC) dot plot is used for the initial setting up of fluorescence detector voltage and cell distribution, R1 region selected viable cells from the suspension and excluded debris from all subsequent dot plots. **B)** R3 is used to select single cells, discarding dublet and triplet cell clumps. **C)** R2 region from the FSC vs PI plot is used to select viable, live cells and exclude PI^+ (dead) cells.

2.2.7 Setting compensation

Compensation for overlapping fluorescence signals (Fig. 2.3) was performed using negative control and single colour controls (tubes 5 – 8, Table 2.1) to obtain true positive expression (see Note 4). For this study, monoclonal antibodies against EpCAM and CD44 were conjugated to phycoerythrin (PE, red) and Alexa Fluor 488 (A488, green) respectively for demonstration of fluorescence compensation. For setting up compensation between these two fluorescence channels, an A488 (X-axis) versus PE (Y-axis) dot plot with quadrant gating was created, with the horizontal line delineating cells positive and negative for PE (R10, Fig. 2.4A, B) and the vertical line for A488 fluorescence (R13, Fig.

2.4C, D). Using the electronic compensation tool from Summit analysis software, sliding scales along the vertical (PE) and horizontal (A488) axes enabled the relevant cell populations to be compensated appropriately to minimise the effect of overlapping fluorescence signals. If compensation is not made, overlapping signals are observed and contribute to a false positive marker expression (R11, Fig. 2.4E, F).

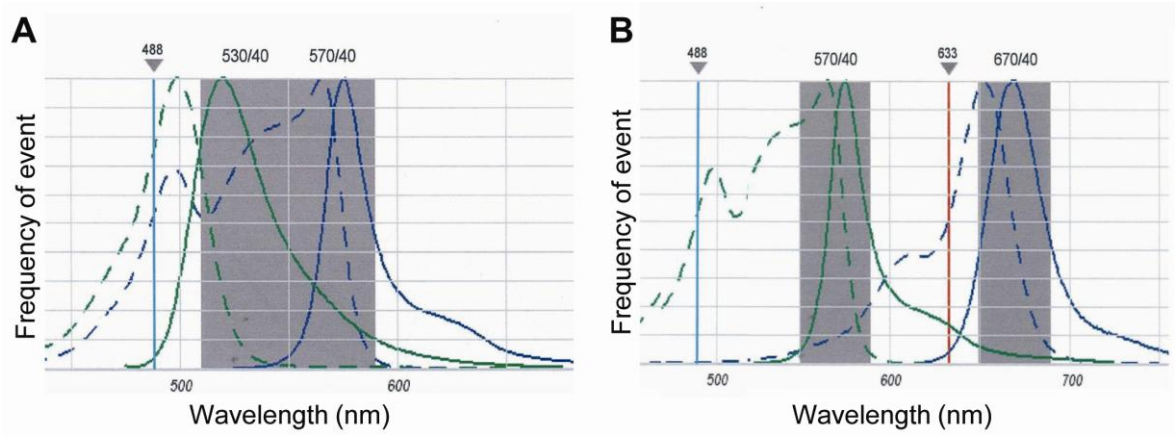


Figure 2.3 Emission spectra of fluorochromes used. A) One excitation laser Arg488 nm is used to excite fluorescence signal from A488 (green line) and PE (blue line) are excited by the (vertical light blue line). **B)** Two different excitation lasers (Arg488 nm for A488 and PE and HeNe633 nm for APC) were used. Band pass filters (530/40, 570/40, 670/40) served to detect peak emission and remove overlapping spectra. In **A)** However, there was still a small overlapping signal that required compensation. A488 and PE peak emission is close to each other, resulting the A488 signal overlaps into PE filter (570/40). Electronic compensation is made to ensure a true PE fluorescence intensity is analysed. In contrast, PE and APC peak emission is further apart from each other **(B)**, no compensation is required. Dotted line, excitation wavelength; solid line, emission wavelength; grey band, band pass filter. Solid blue and red vertical line depicted excitation laser Arg488 and HeNe633 respectively. PE, phycoerythrin; A488, Alexa Fluor 488; APC, Allophycocyanin. Adapted from Invitrogen fluorescence spectra (www.invitrogen.com).

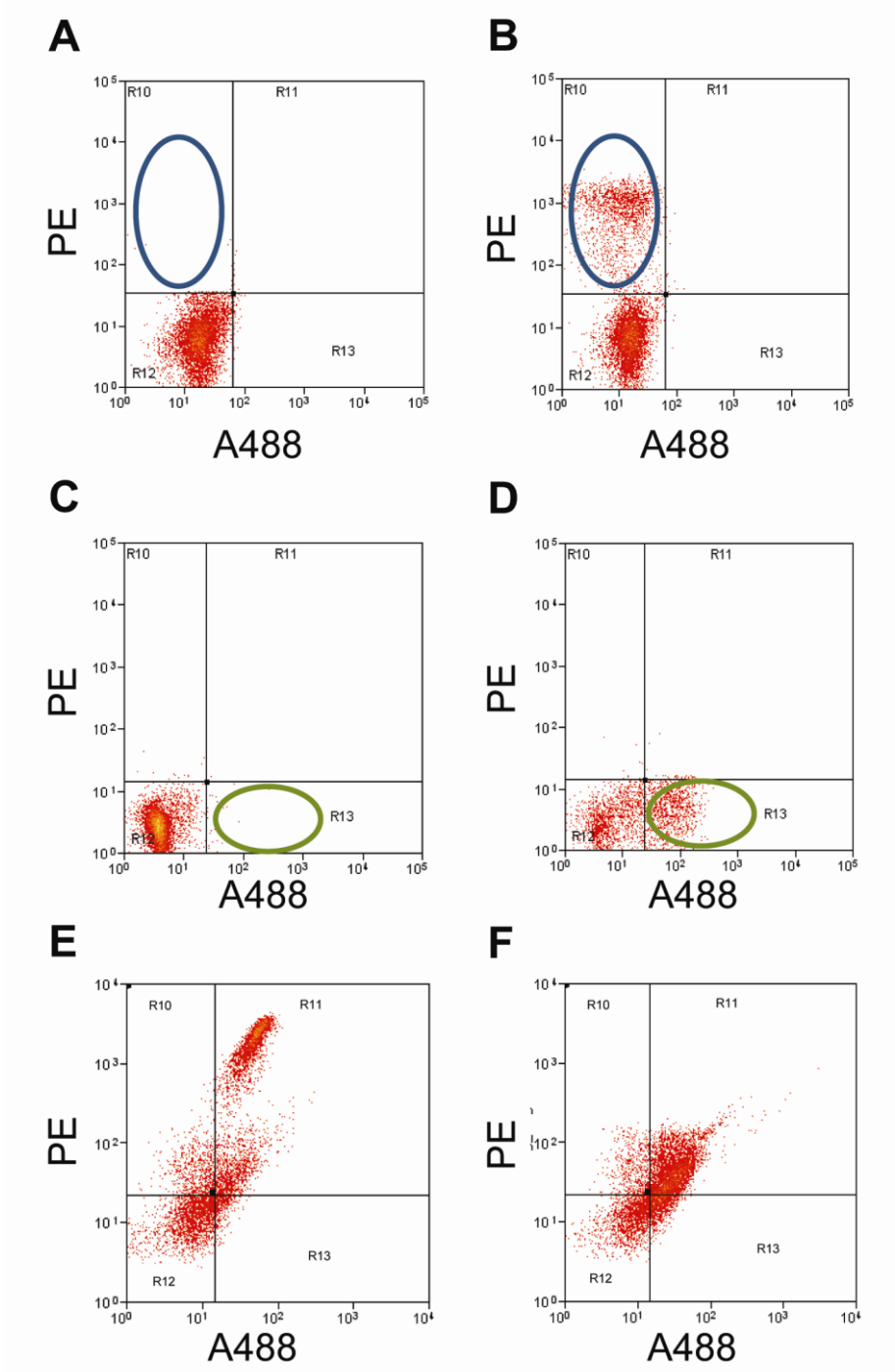


Figure 2.4 Flow cytometry profile of human endometrial epithelial single cell suspensions required to set up compensation. Two-colour dot-plots of epithelial cells stained with **A, C)** isotype IgGs and **B, D)** single-colour controls, required to set up **A-B)** vertical gating for PE (R10 region). **C-D)** horizontal gating for A488 (R13 region). **E-F)** False positive marker expression (R11 region) results if compensation was not made.

Following compensation, at least 5,000 cells from the sample tube (tube 9, Table 2.1) were acquired prior to sorting to obtain a representative percentage of cells positive for marker expression for PE and A488 fluorescence. Endometrial epithelial cells were positively selected by electronic gating of EpCAM-PE positive/CD90 negative cells (R11, Fig. 2.5A), which was combined with previously set electronic gating (R1, R2, R3 on Fig. 2.2) to remove debris, multiple cells and dead cells. Once these parameters were set, cell acquisition for analysis and/or sorting commenced. Re-analysis of the sorted pure endometrial epithelial cells showed significant purification of epithelial cells compared with before sorting (Fig. 2.5B, C).

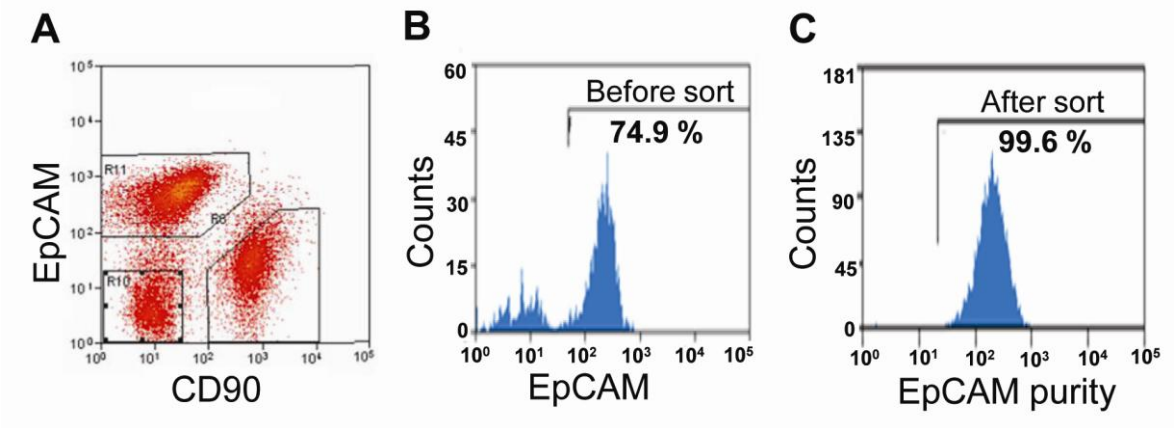


Figure 2.5 Flow cytometric analysis of EpCAM expression in endometrial epithelial single cell suspensions. A) R11 showed EpCAM positive expressing cells, R8 and R10 negatively select other contaminating cells including CD90 positive (stromal-like cells). **B)** Histogram plot indicating expression level of EpCAM-positive cells after gating on different regions with respect to negative controls. This plot showed that 74.9% of epithelial cells were reactive to EpCAM. **C)** Quality control of EpCAM-positive cells after the completion of flow cytometry sorting, showing 99.6% of cells purity.

For culture of sorted endometrial epithelial cells, cells were sorted into sterile tubes containing cold buffered collection medium (DMEM/F12-HEPES, 1% antibiotics, 10% FCS) sitting in a cooled tube-holder attached to the cell sorter. It is important to note that all tubes were maintained at 4ºC throughout the cell sorting and before seeding onto culture plates in order to minimise cell death (see Note 3).

2.2.8 Colony formation of sorted human endometrial epithelial cells

Mitomycin-C inactivated endometrial stromal cells were pre-seeded onto fibronectin-coated plates at least 4 hr prior to seeding sorted endometrial epithelial cells (see Note 5 and 6), to allow the stromal feeder layer to attach. Prior to seeding of the sorted epithelial cells, the stromal culture medium was removed and replaced with pre-warmed epithelial culture medium (sodium bicarbonate buffered DMEM/F-12, primocin, glutamine and 50 ng/ml EGF).

Sorted tubes containing epithelial cells were centrifuged for 4 min at 209 g at 4ºC, media was removed, and cells resuspended in 200 µl of epithelial culture medium, carefully handling the pellet, as it was often very small and invisible to the naked eye. A cell count was performed for each sorted sub-population and the sorted epithelial cells seeded at clonal density (50 and 100 cells/cm^2) in duplicates on stromal feeder plates prepared earlier (see Note 7). Clonal cultures were allowed to grow in a humidified incubator (5% CO_2) at 37ºC for 14 days. Colony formation was observed microscopically to ensure colonies were derived from single cells, and medium was changed every 6 - 7 days. Clonal cultures were terminated between 13 - 17 days as previously described (Chan *et al.*, 2004).

2.2.9 Visualisation of endometrial epithelial colonies

Clonal epithelial culture plates were washed twice with PBS, fixed with 5 ml of 10% buffered formalin (performed inside fume hood) for 10 min at RT, washed thrice with PBS, and incubated with filtered Harris haematoxylin (Amber Scientific, WA, Australia) for 4 min at RT. The haematoxylin stained colonies were then washed twice with running tap water, once with Scott's tap water (NH_4^+ in water 1:10 ratio) to develop the blue colony staining, and washed twice more with running tap water as before. Plates were allowed to dry overnight before being assessed for colony growth. Colonies (> 50 cells) were counted using Bio-Rad ChemiDoc XRS Molecular Imager and analysed using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

2.2.10 Three-dimensional (3D) culture of epithelial clonally derived cells

This part of the study was previously optimised and has been published (Gargett *et al.*, 2009). A brief protocol outlined below is recommended for investigating 3D culture of flow cytometry sorted endometrial epithelial cells (see Note 8).

Firstly, endometrial stromal cells were cultured as a monolayer in 8-well chamber slides (BD Biosciences) for at least 24 hr prior to seeding sorted epithelial cells. 100% purified Matrigel™ (BD Biosciences) was thawed on ice, the culture media removed from the chamber slides, and 50 µl containing $2x10^5$ of sorted epithelial cells was gently mixed with 50 µl of Matrigel™ before being transferred to the prepared chamber slides. A volume of 200 µl of epithelial culture medium was added to each well of the chamber slide. Epithelial cells were allowed to grow in a 5% CO_2 humidified incubator at 37ºC for at least 25 days (Gargett *et al.*, 2009). Medium was changed every 3 - 4 days by removing half the volume

of the old media and replacing with pre-warmed medium. Microscopic observation was performed every 1 – 2 days to observe for ball-like and gland-like structures. Gland-like structures were further characterised as described (Gargett *et al.*, 2009).

2.3 Notes and recommendations

1. At the beginning of this study, endometrial tissue was dissociated to endometrial glands and subsequent single cell suspensions using DNase I (4 mg/ml) and collagenase type III (2.5 mg/ml in PBS). However, this was found to negatively affect epithelial cell viability (Supplemental Fig. 2.1A, E). The type of collagenase and the duration of endometrial tissue dissociation are very important. It was found that collagenase type I contained less contaminating trypsin and was therefore less damaging than collagenase type III in releasing stromal and epithelial cells from the tissue (Worthington Biochemical, Tissue Culture Handbook). The time taken for the initial tissue dissociation should be carefully monitored microscopically and will vary depending on the size and menstrual stage of the tissue sample. An overly long dissociation time could potentially break down all glandular epithelium in the first dissociation at the expense of cell viability (Chaminadas *et al.*, 1986). Furthermore, longer exposure times to enzyme treatment could damage cell surface antigens and reduce the antibody binding during immunostaining for flow cytometry sorting, lowering cell yields (Shmelkov *et al.*, 2008; Visvader & Lindeman, 2008).
2. Trypsin-EDTA should not be used to disperse glandular epithelium to single cell suspension during the second dissociation, due to its harsh effects on endometrial epithelial cell viability, thus limiting the ability of cells to adhere to culture plates

(Supplemental Fig. 2.1 B, D) (Viganò *et al.*, 1993). Alternatively, collagenase II yielded a better epithelial single cell suspension compared to trypsin-EDTA. Furthermore, it was imperative that the second dissociation step does not exceed 30 min, as this also causes cellular damage. Gland fragments that break down after 30 min of digestion were likely from secretory functionalis glands containing mostly differentiated cells that undergo apoptosis if culture is attempted (Gargett *et al.*, 1999).

3. It cannot be stressed strongly enough the importance of ensuring that the tube holder is at 4ºC during flow cytometry sorting (this may not be available in some machines, thus ice should be used), since temperature fluctuations increase cellular damage (Kirk *et al.*, 1978). Additionally, the medium for collecting sorted cells should be pH-stable and adequately buffered with HEPES or similar and containing 10% serum.

4. Compensation for multiple combinations of fluorochromes is very important due to spectral overlap. Compensation ensures that a true cell population is analysed and acquired. The choice of fluorochromes while dependant on availability of reagents, the limitations of the flow cytometer instrument is also important. The most commonly used fluorochromes are fluorescein isothiocyanate (FITC or Alexa Fluor 488, A488), phycoerythrin (PE), and propidium iodide (PI). However, this combination has a wide range of spectral overlap in energy emission and excitation spectra. Compensation is necessary for the A488 and PE combination and for the PE and PI combination. Compensation and true positive expression can be obtained by using different combination of fluorochromes (for example PE/A647/7-aminoactinomycin D (7-AAD), or the biotin-avidin system or a tandem-dye conjugated fluorochrome, where the spectra do not overlap). Dead cells and debris

can also be removed using 7-AAD or SYTOX® Blue instead of PI. This selection is dependent on the choice of combination of fluorochromes and the capability of the flow cytometer or sorter, since SYTOX® Blue requires a violet laser (405 nm). Further information on fluorochromes, filter combination and laser types can be found on the Invitrogen online tutorial at www.invitrogen.com/handbook/spectraviewer.

5. Stromal feeder layers can be prepared 24 hr prior to seeding sorted epithelial cells. Unused mitomycin-C inactivated stromal cells can be stored for at least 3 months in liquid nitrogen, however, a careful freeze-thaw cycle should be adopted to ensure good cell yield. Stromal feeder layers supported clonal growth of flow cytometry sorted endometrial epithelial cells. Previous findings have suggested that estrogen induces epithelial cell proliferation via estrogen receptors located on stromal cells which in turn act on the epithelial cells (Cooke *et al.*, 1997). To study this effect, epithelial cell growth was examined in stromal-conditioned medium, pre-treated with 1 nM or 10 nM estrogen for 24 or 48 hr (Supplemental Table 2.1). It was observed that stromal-conditioned medium did not enhance epithelial cell growth. To our surprise, the stromal-conditioned medium induced the epithelial cells to undergo differentiation, resulting in mature epithelial cell phenotype or a stromal-like morphology (Supplemental Fig. 2.2B-D) (Bongso *et al.*, 1988). This study and that of others showed the importance of direct epithelial-stromal interactions in providing the niche for stem cell proliferation and maintenance (Blauer *et al.*, 2005; Gargett *et al.*, 2009). Furthermore, this suggests that epithelial cells may require cell-bound growth factors obtained from direct contact between epithelial cells with

stromal feeder cells which is lacking in stromal-conditioned medium (Zhang *et al.*, 1995).

6. Both fibronectin and matrigel supported epithelial cell attachment and growth. Epithelial cells grown on Matrigel™ matrix were small and loosely packed (Supplemental Fig. 2.2E, F). The best epithelial cell growth occurred on fibronectin coating, producing a more defined large, tightly packed colony of small cells confirming previous reports, showing that extracellular matrices were required to maintain epithelial cell cultures (White *et al.*, 1990; Chan *et al.*, 2004).

7. Epithelial culture medium supplemented with epidermal growth factor (EGF) and 10% fetal calf serum was the best culture medium for endometrial epithelial cells in this study. This agrees with previous findings that EGF supports endometrial epithelial clonogenic activity (Chan *et al.*, 2004; Schwab *et al.*, 2005; Gargett, 2006; Gargett *et al.*, 2008). EGF stimulates epithelial cell proliferation by signalling through the EGF receptor (Nelson *et al.*, 1991; Smith *et al.*, 1991; Zhang *et al.*, 1995). EpiLife®, a commercially available medium routinely used to culture epidermal keratinocytes (Invitrogen – Cell culture handbook) was not suitable for the culture of endometrial epithelial cells.

8. A previous report has shown that endometrial epithelial clonally derived cells differentiate into cytokeratin-expressing gland-like structures (Gargett *et al.*, 2009). Thus the protocol described here can be used to examine the ability of flow cytometry sorted epithelial cells to form 3D gland-like structures. The technical challenge of this protocol is the manual handling of Matrigel™ which is semi-solid at room temperature. To maintain a semi-liquid form, Matrigel™ should be kept on ice and should be transferred using refrigerated pipette tips.

2.4 Summary

In this study we have shown that multiple factors contributed to successful clonal cultures of sorted endometrial epithelial cells. It took approximately 8 - 10 hr to isolate, dissociate and sort endometrial epithelial cells, thus the choice of enzymes, the duration of tissue dissociation greatly impacted on epithelial cell survival. While flow cytometry is robust in cell separation and sorting cells into pure populations, its long duration causes cellular damage, limiting cell survival for establishing clonal cultures. Stromal feeder layers supported sorted epithelial cell clonal cultures better than stromal-conditioned medium. An alternative approach to flow cytometry sorting is magnetic bead sorting, which is much simpler and requires shorter time. However, it is beyond the scope of this study and is discussed in Chapter 4. In addition, the choice of surface marker also plays a role in ensuring a true cell population is obtained. In this study, we used EpCAM to isolate a purified endometrial epithelial population while gating out contaminating stromal cells ($CD90^+$ cells). Compensation was also necessary to minimise fluorescence spectra overlap which contributes to false positive marker expression. The protocol reported here will allow further characterisation of endometrial epithelial cells by enabling examination of candidate surface markers of endometrial epithelial stem/progenitor cells.

2.5 Supplementary Information

Preparation of stromal conditioned medium

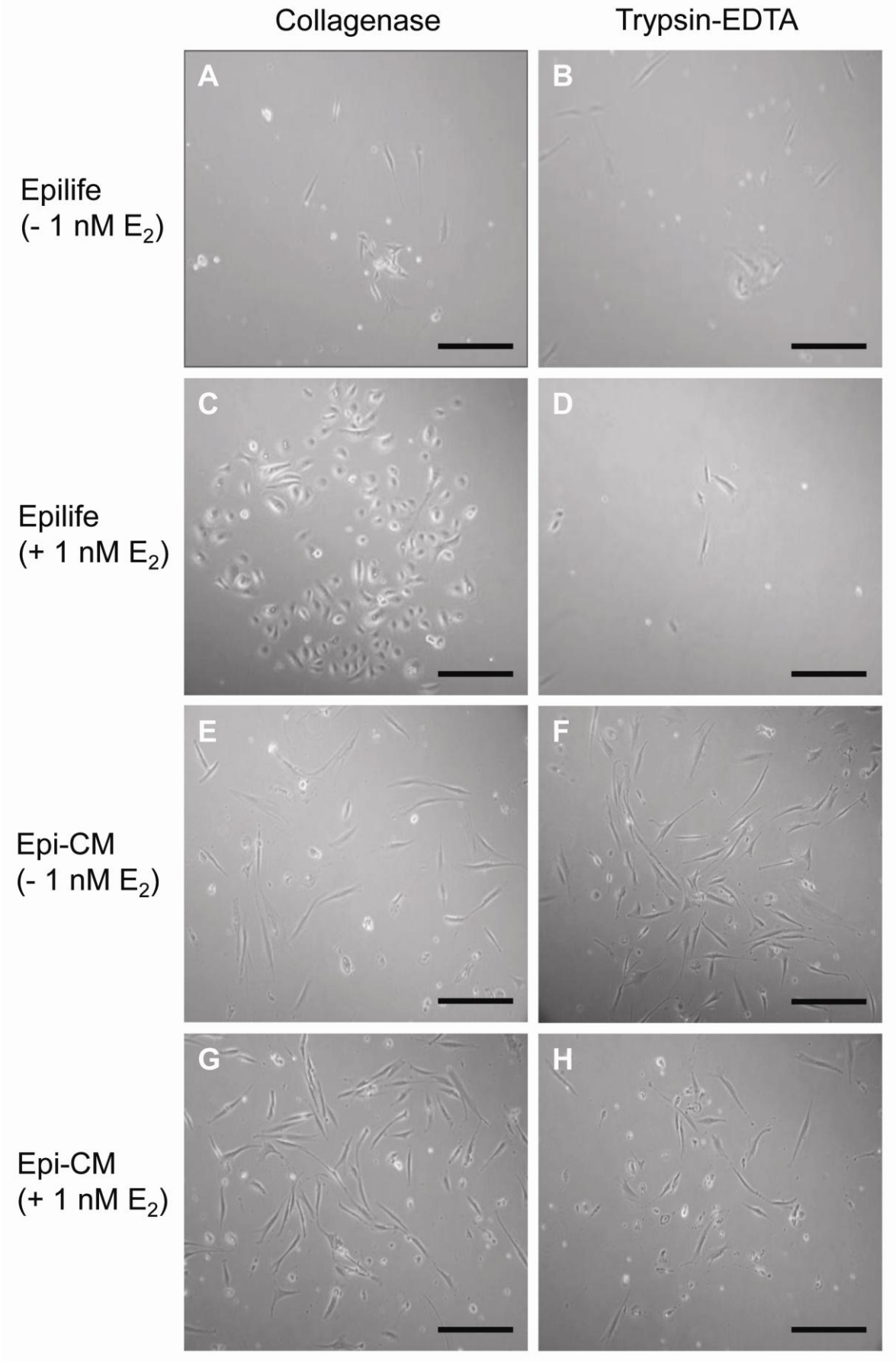
Prepare regents required to obtain serum-free stromal conditioned medium as tabulated below. This protocol has been adopted, modified and optimised based on several published works (Zhang *et al.*, 1995; Chan *et al.*, 2004; Punyadeera *et al.*, 2008).

Supplemental Table 2.1 Reagents required for preparation of serum-free stromal conditioned medium

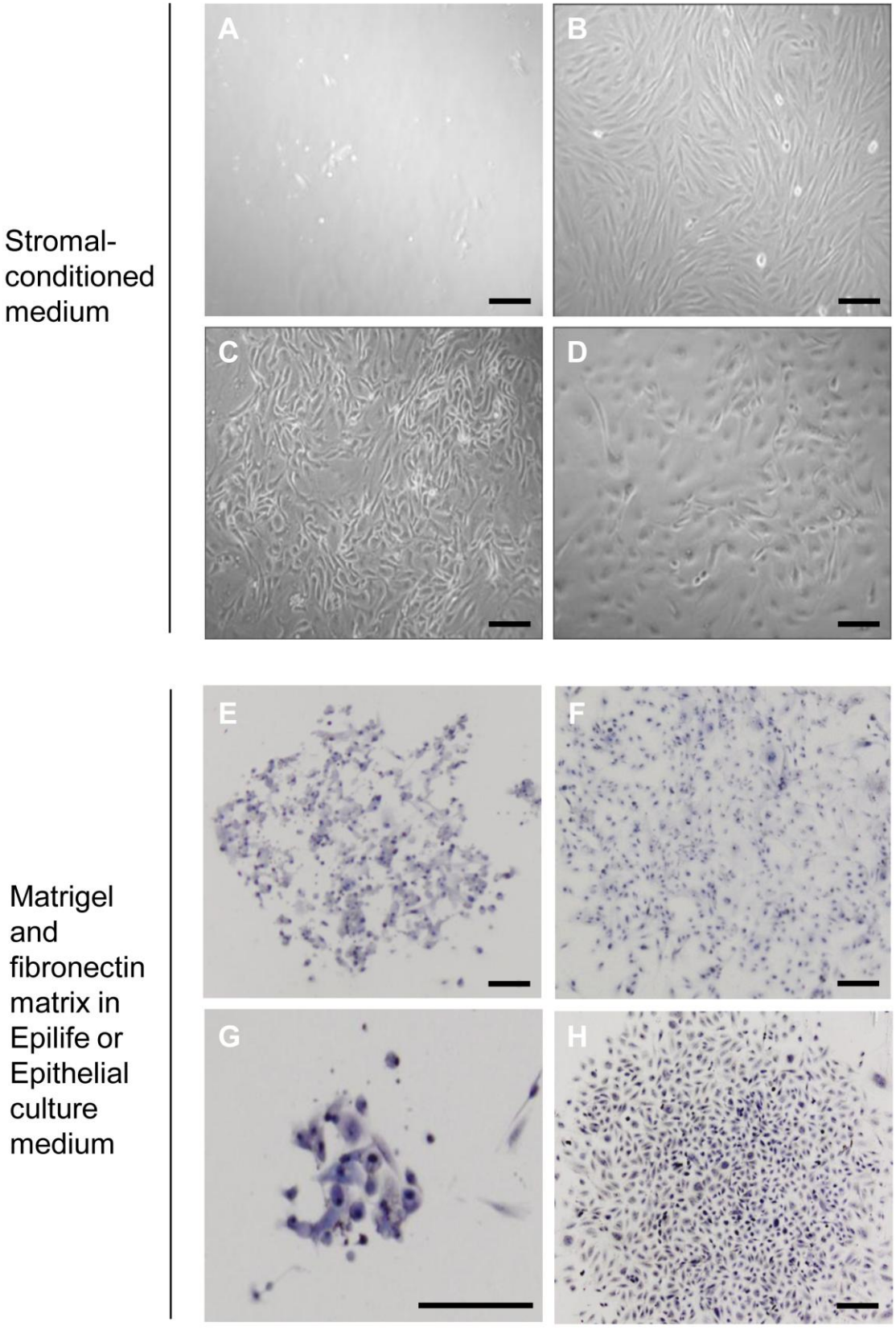
Components	Volume (ml) to make 100 ml	Sources
Sodium biocarbonate buffered DMEM/F12	85 ml	Invitrogen
Antibiotic-Antimycotic	1 ml	Invitrogen
Glutamine	1 ml	Invitrogen
100x Non-Essential Amino Acids (NEAA)	1 ml	Invitrogen
100x Insulin-Transferrin-Selenium (ITS)	1 ml	Invitrogen
55mM B-Mercaptoethanol	184 µl	Invitrogen
100 µg/ml EGF	10 µl	Invitrogen
100 µg/ml bFGF	10 µl	Invitrogen
5% bovine serum albumin (BSA)	10 ml	Sigma Aldrich
10 mg/ml heparin	1 ml	Stem Cell Technologies
100 mM L-Ascorbic acid-2-phosphate	100 µl	Sigma Aldrich
50 µM Linoleic acid	20 µl	Invitrogen
1 nM or 10 nM estrogen	1µl	Sigma Aldrich

Stromal monolayer grown in T175 flasks (BD Biosciences) were incubated with serum-free medium supplemented with estrogen for 24 and/or 48 hr in a humidified 5% CO_2 incubator. After the incubation, medium was collected and centrifuged for 10 min at 440 g. The medium was filter sterilised using 0.22 µm filter and stored in 1 ml aliquots at -20^{o}C until use.

Supplemental Figure 2. 1 Phase contrast images of human endometrial epithelial cells isolated by different enzymes and cultured in various culture media. Endometrial tissue was dissociated using **A, C, E, G)** DNase I/collagenase III/collagenase II protocol and **B, D, F, H)** DNase I/collagenase III/ trypsin-EDTA protocol. Endometrial epithelial cell were cultured in EpiLife® culture medium without (A, B) and with (C, D) 1 nM estrogen, epithelial culture medium containing 2.5% fetal calf serum (Epi-CM) in the absence (E, F) and presence (G, H) of estrogen. C, collagenase; T, trypsin-EDTA; E_2, estrogen. Scale bar, 10µm.


Collagenase
Trypsin-EDTA
Epilife
(- 1 nM E_2)
Epilife
(+ 1 nM E_2)
Epi-CM
(- 1 nM E_2)
Epi-CM
(+ 1 nM E_2)
A
B
C
D
E
F
G
H

Supplemental Figure 2.2 The effects of stromal-conditioned medium and matrices on clonal endometrial epithelial cell adherence and growth. Phase contrast images of epithelial cells cultured in **A)** serum-free medium, showing no cell attachment and lack of survival. **B)** epithelial-mesenchymal-like transition in the presence of stromal conditioned medium obtained from stromal cells, pre-treated with 1 nM estrogen for 24 hr. **C-D)** whorl-like epithelial cells in the presence of stromal conditioned medium obtained from stromal cells, pre-treated with 10 nM and 1 nM estrogen for 24 hr and 48 hr respectively. Haematoxylin staining of fixed epithelial cell colony grew on **E-F)** Matrigel™ matrix and **G-H)** fibronectin matrix in EpiLife® medium (E, G) or epithelial culture medium (F, H). Scale bar, 10 µm.


Stromal-conditioned medium
A
B
C
D
Matrigel and fibronectin matrix in Epilife or Epithelial culture medium
E
F
G
H

Chapter 3

Differential expression of Wnt signalling molecules between pre- and post-menopausal endometrial epithelial cells suggests a population of putative epithelial stem/progenitor cells reside in the basalis layer

Declaration

Monash University

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Responsible for refining the research question, performing the experiments, collecting and analysing and generating data for all figures except Figure 1, and manuscript writing	80 %

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Carl Sprung	Performed the initial microarray data analysis from raw data and helped revise the manuscript	10 %
Caroline Gargett	Conception and design of the study, helped revise the manuscript, read and approved the final manuscript	10 %

Candidate's Signature	[redacted]	Date 9 Dec 2011

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	The Ritchie Centre, Monash Institute of Medical Research Department of Obstetrics and Gynaecology, Monash University Monash Medical Centre, 246 Clayton Road, Clayton, Victoria, 3168, Australia

[Please note that the location(s) must be institutional in nature, and should be indicated here as a department, centre or institute, with specific campus identification where relevant.]

Signature 1	[redacted]	**Date** 9/12/11
Signature 2		9/12/11
Signature 3		

Abstract

The human endometrium undergoes extensive monthly regeneration in response to fluctuating levels of circulating estrogen and progesterone in pre-menopausal (Pre-M) women. In contrast, post-menopausal (Post-M) endometrium is thin and quiescent with low mitotic activity, similar to the Pre-M endometrial basalis layer. Clonogenic epithelial stem/progenitor (ESP) cells likely responsible for regenerating endometrial epithelium have been identified in Pre-M and Post-M endometrium but their location is unknown. We undertook transcriptional profiling of highly purified epithelial cells from full-thickness Pre-M and Post-M endometrium to identify differentially regulated genes that may indicate a putative ESP cell population resides in the basalis of Pre-M and basalis-like Post-M endometrium. Of 1,077 differentially expressed genes identified, the Wnt signalling pathway, important in endometrial development and stem cell regulation, was one of the main gene families detected, including 22 Wnt-associated genes. Twelve genes were validated using quantitative RT-PCR and all were concordant with microarray data. Immunostaining showed glandular epithelial location of Wnt-regulated genes, Axin2 and β-catenin. Axin2 localised to the nucleus of basalis Pre-M and Post-M and cytoplasm of functionalis Pre-M endometrium, suggesting that it regulates β-catenin. Comparison of our Post-M gene profile with published gene microarray datasets revealed similarities to Pre-M basalis epithelial profiles. This differential expression of multiple Wnt-associated genes in human Pre-M and Post-M endometrial epithelial cells and the similar gene profile of Post-M and Pre-M basalis epithelium suggests that a population of putative endometrial ESP may reside in the basalis of Pre-M endometrium, which may be responsible for regenerating glandular epithelium each month.

3.1 Introduction

Pre-menopausal endometrium is highly regenerative undergoing more than 400 cycles of regeneration, differentiation and shedding during a woman's reproductive years (Jabbour *et al.*, 2006). Full-thickness endometrium consists of the functionalis and basalis layers (Fig. 3.1A) and is responsive to fluctuating levels of circulating ovarian steroid hormones, estrogen and progesterone (Jabbour *et al.*, 2006; Gargett, 2007). During menstruation, the functionalis is shed while the basalis remains, and 4-10 mm of new functionalis is regenerated in the following cycle. In contrast, Post-M endometrium is thin, quiescent and atrophic (Fig. 3.1A) with low mitotic activity, and is thought to be similar to the basalis of Pre-M endometrium (Gargett, 2007; Kurman *et al.*, 2011). As circulating estrogen is very low in post-menopausal women, the functionalis is virtually absent. However, when hormone replacement therapy is given, Post-M endometrium can regenerate sufficiently to support pregnancy to term (Paulson *et al.*, 2002; Klaassens *et al.*, 2006).

Given the monthly tissue remodelling of the functionalis, it has been postulated that resident epithelial stem/progenitor (ESP) cells are located in the basalis and are responsible for its remarkable regeneration (Padykula *et al.*, 1989; Padykula, 1991; Gargett, 2007). This concept was strengthened by a kinetic study showing lower cellular proliferation rates in the basalis and in Post-M endometrium compared to the functionalis (Ferenczy *et al.*, 1979). Evidence for the existence of endometrial ESP cells was first demonstrated by the identification of rare clonogenic epithelial cells in Pre-M and in atrophic, inactive Post-M endometrium (Chan *et al.*, 2004; Schwab *et al.*, 2005; Gargett *et al.*, 2009), however the precise location of clonogenic ESP cells is unknown.

Gene expression profiling of Pre-M endometrium across the menstrual cycle has been

studied previously using fresh unfractionated endometrial tissue (Ponnampalam *et al.*, 2004; Yanaihara *et al.*, 2004; Yanaihara *et al.*, 2005; Talbi *et al.*, 2006). Differential expression of genes between functionalis and basalis epithelial cells has been identified in menstrual endometrium in a laser capture microdissection study (Gaide Chevronnay *et al.*, 2009; Gaide Chevronnay *et al.*, 2010) and between glandular and luminal epithelial compartments in mice (Niklaus & Pollard, 2006). Studies using a mouse model have shown that the Wnt signalling pathway is essential for female reproductive tract development (Miller *et al.*, 1998; Parr & McMahon, 1998). Gene profiling studies of endometrial tissue showed that the Wnt signalling pathway was regulated by steroid hormones during its cycles of growth and differentiation (Bui *et al.*, 1997; Tulac *et al.*, 2003; Hou *et al.*, 2004; Tulac *et al.*, 2006). In human endometrium, Wnt2, Wnt3, Wnt4, Wnt5, Wnt7a and Wnt7b were expressed in both proliferative and secretory stages of the menstrual cycle (Bui *et al.*, 1997; Tulac *et al.*, 2003). Wnt receptor and co-receptors (*FZD6, LRP6*) and downstream effectors (*DKK1, DVL-1, GSK3β* and *β-catenin*) were also expressed throughout the menstrual cycle (Tulac *et al.*, 2003). Wnt signalling is a critical downstream mediator of estrogen-induced endometrial proliferation (Hou *et al.*, 2004). Progesterone counteracts this proliferative effect by inducing differentiation (Tulac *et al.*, 2006; Wang *et al.*, 2009; Wang *et al.*, 2010). While these studies highlight the key role of Wnt signalling in endometrial function, there are very few investigations specifically focused on human endometrial luminal and glandular epithelial cells.

This study aimed to investigate the transcriptional profiles of purified epithelial cells isolated from full-thickness Pre-M and basalis-like Post-M endometrium in order to elucidate differences between the epithelium of the rapidly remodelling functionalis and the quiescent basalis. In this experimental design, functionalis epithelium will dominate the

gene expression profile of full-thickness Pre-M endometrium, while the Post-M endometrial epithelial gene profile will be similar to the epithelium of the quiescent Pre-M endometrial basalis. Differential gene expression of many Wnt family members was identified. Comparative analysis of our endometrial epithelial gene expression profiles to that of endometrial epithelial cells in remodelling endometrium (Gaide Chevronnay *et al.*, 2009; Gaide Chevronnay *et al.*, 2010) also provides new evidence that Post-M endometrial epithelium has a similar gene signature to that of basalis epithelium of menstrual endometrium. Our data suggests that the Wnt signalling pathway may have a role in the function of a population of putative ESP cells possibly located in Post-M and the basalis layer of Pre-M endometrium.

3.2 Material and Methods

3.2.1 Patients

Human endometrium attached to the underlying myometrium (n = 40: 13 proliferative, 12 secretory and 15 post-menopausal) were obtained from women undergoing hysterectomy for benign gynaecologic conditions including menorrhagia, prolapse, fibroids, endometriosis and adenomyosis (Table 3.1). Ethical approval was obtained from Southern Health Human Research & Ethics Committee B and Monash University Human Research Ethics Committee with informed written consent given by each patient. Histo-pathological evaluation and menstrual cycle dating was assessed according to well-established criteria (Noyes *et al.*, 1975) (Fig. 3.1B). Inclusion criteria were normal cycling pre-menopausal women (30 to 53 yr old) with benign endometrium who had not taken steroid hormone treatment within 3 months prior to surgery and post-menopausal (49 to 70 yr old) women, who had not menstruated for at least 1 year nor had taken hormones for 3 months prior to

surgery. Exclusion criteria were women undergoing hysterectomy for endometrial pathologies; polyps, hyperplasia or endometrial cancer.

3.2.2 Endometrial tissue dissociation

The endometrium including the non-menstruated basalis layer was scraped and dissected from the superficial myometrium to ensure collection of all endometrial tissue as previously described (Chan *et al.*, 2004; Gargett *et al.*, 2009). In Pre-M samples, it was more consistent to remove the entire endometrium comprising functionalis (80-90%) and basalis (10-20%) for comparison with Post-M endometrium than to attempt gross separation of functionalis and basalis on the uterine tissue pieces (~10 x 10 x 5 mm) made available by the pathologist. Using this reliable, robust method, we were able to consistently obtain samples suitable for analysis. Endometrial tissue was then minced finely and digested in culture medium (Sodium bicarbonate buffered DMEM-F12, 10% fetal calf serum, 1% antibiotics and 1% glutamine, Invitrogen, Carlsbad, CA, USA), 5 mg/ml of collagenase I and 40 µg/ml deoxyribonuclease I (Worthington Biochemical, Freehold, NJ, USA) using a MACSmix rotator (Miltenyi Biotec, Bergisch Gladbach, Germany) at 37ºC for 90 min in a 5% CO_2 humidified incubator. The digestion was stopped and filtered through a 40 µm sieve (BD Biosciences, Durham, NC, USA). Glandular fragments containing epithelial cells remaining on the filter were backwashed and further digested for 20 min in 0.8 mg/ml collagenase II (Worthington) and 40 µg/ml deoxyribonuclease I. Epithelial cell suspensions were then filtered through a 40 µm sieve (BD Biosciences) and centrifuged for 5 min at 209 g and the pellets were resuspended in 5 ml medium. Cell counts were performed using a haemocytometer.

TABLE 3.1 Details of patient samples used in this study

Sample Number	Age	Menstrual stage	Indication/Diagnosis	Assay
1	48	Proliferative	Menorrhagia	M
2	52	Proliferative	Prolapse	M, Q
3	49	Proliferative	Menorrhagia	M, Q
4	52	Proliferative	Fibroids	M, Q
5	45	Secretory	Menorrhagia	M, Q
6	48	Secretory	Fibroids	M, Q
7	30	Secretory	Severe endometriosis	M, Q
8	48	Secretory	Menorrhagia	M, Q
9	55	Post-menopausal	Fibroids	M
10	57	Post-menopausal	Prolapse	M
11	49	Post-menopausal	Unknown	M, Q
12	47	Proliferative	Menorrhagia	Q
13	36	Proliferative	Menorrhagia	Q
14	49	Proliferative	Fibroids	Q
15	40	Proliferative	Fibroids	Q
16	41	Secretory	Prolapse	Q
17	41	Secretory	Prolapse	Q
18	47	Secretory	Prolapse	Q
19	46	Secretory	Menorrhagia	Q
20	53	Post-menopausal	Prolapse	Q
21	54	Post-menopausal	Unknown	Q
22	64	Post-menopausal	Prolapse	Q
23	63	Post-menopausal	Prolapse	Q
24	53	Post-menopausal	Prolapse	Q
25	65	Post-menopausal	Prolapse	Q
26	66	Post-menopausal	Prolapse	Q
27	50	Post-menopausal	Prolapse	Q
28	54	Post-menopausal	Prolapse	Q
29	43	Proliferative	Menorrhagia	I
30	53	Proliferative	Adenomyosis	I
31	49	Proliferative	Adenomyosis	I
32	51	Proliferative	Menorrhagia	I
33	46	Proliferative	Menorrhagia	I
34	39	Secretory	Menorrhagia	I
35	38	Secretory	Adenomyosis	I
36	48	Secretory	Menorrhagia	I
37	43	Secretory	Adenomyosis	I
38	53	Post-menopausal	Prolapse	I
39	65	Post-menopausal	Prolapse	I
40	70	Post-menopausal	Prolapse	I

M, Microarray; Q, qRT-PCR; I, Immunofluorescence

3.2.3 Endometrial epithelial cell selection and RNA extraction

Anti-human EpCAM (BerEP4 clone) antibody-coated magnetic Dynabeads (Dynabeads Epithelial Enrich kit, Invitrogen) was used to positively select both luminal and glandular epithelial cells from cell suspensions (Chan *et al.*, 2004). Briefly, washed epithelial Dynabeads (4 beads/cell) were incubated with the cells at 4ºC with gentle mixing for 30 min. The cell-bead suspension was placed on the magnet for 5 min to select beaded cells (Fig. 3.1C), washed twice and the beaded epithelial cells were resuspended in PBS. Using established protocol (Gargett *et al.*, 2009), the purity of bead-selected epithelial cells was confirmed by positive staining for the epithelial marker, cytokeratin (> 99.8%) and negative for stromal maker, CD90 (Supplemental Fig. 3.1). Total RNA was extracted from bead-purified epithelial cells using Ambion RNAqueous Micro Kit (Ambion, Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. RNA preparations were DNase treated and purified using DNase inactivation kit (Ambion). RNA purity and integrity was analysed by A260/A280 nm ratio using a NanoDrop ND1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA) and stored at -80ºC until use.

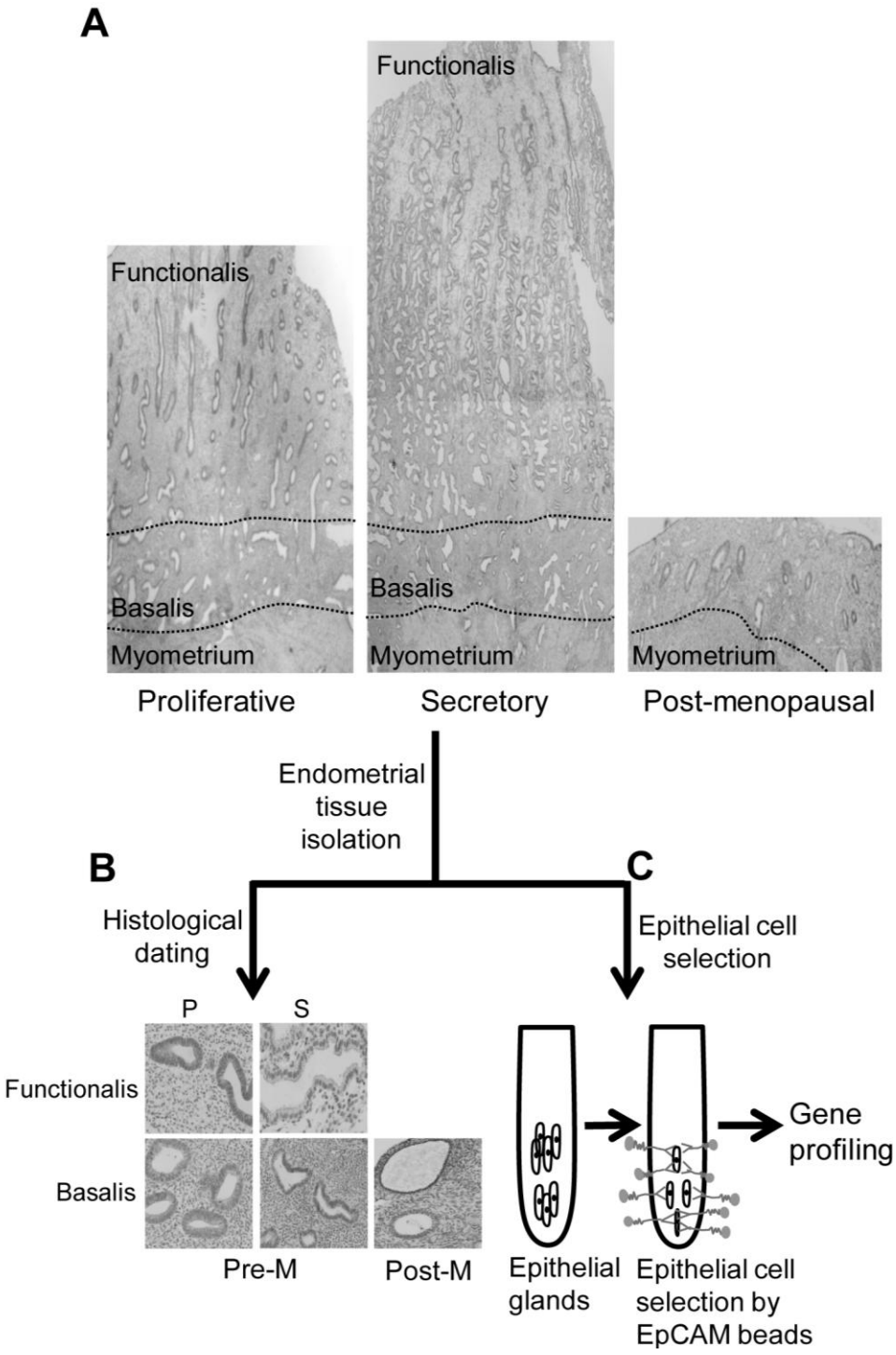


Figure 3.1 Experimental flow chart for investigating epithelial cell gene expression in Pre-M and Post-M human endometrium. (A) Full-thickness endometrium showing functionalis and basalis layers in Pre-M endometrium (proliferative, P and secretory, S stage) and thin, atrophic, basalis-like Post-M endometrium. (B) Histological dating and glandular epithelial morphology of Pre-M (P and S) and Post-M endometrium. (C) Epithelial cells of Pre-M and Post-M endometrium were dissociated and purified using magnetic bead sorting with EpCAM-labelled beads for subsequent transcriptome studies.

3.2.4 Gene expression profiling

Gene chip hybridisation, scanning and data acquisition was performed at the Australian Genome Research Facility (AGRF) (Melbourne, Australia). Briefly, RNA samples (n = 11) (Table 1) were assessed for quality and integrity using Agilent Bioanalyser 2100 (Agilent, Santa Clara, CA, USA). Samples (500 ng) were labelled using the Ambion total prep RNA amplification kit. A total of 1.5 µg of labelled cRNA was used to prepare a probe cocktail (0.05 µg/µl cRNA and GEX-HYB hybridisation buffer according to the manufacturer's protocol) for hybridisation to the Sentrix Human-HT12 v3 Beadchip (Illumina, San Diego, CA, USA). The chip was hybridised at 58ºC for 16 hours on a rocking platform, washed, coupled with Cy3 and scanned using the Illumina BeadArray Reader. BeadStudio scanner software (Illumina) was used to convert signal on the array to an appropriate file for analysis.

3.2.5 Microarray data analyses

The intensity of probe sets and batch normalisation were analysed using Partek Genome Suite 6.5 analysis package (Partek, St Louis, MO, USA). Robust Multichip Average (RMA) (a computerised statistical package) algorithm and analysis of variance (ANOVA) were applied to adjust background noise, quantile normalisation and data summarisation (Bolstad *et al.*, 2003; Sprung *et al.*, 2011). All raw and processed data files have been deposited in the National Centre for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) Dataset with accession number GSE35221.

Comparison was made between 3 Post-M samples and 8 Pre-M (4 proliferative and 4 secretory). Principal component analysis (PCA) and hierarchical clustering were performed using Partek software. After RMA normalisation, the PCA algorithm in Partek was applied

to all 11 samples to identify trends in the data and as a quality control measure. Differential gene expression and hierarchical clustering of the Wnt-associated genes was generated from comparison between Post-M and Pre-M (proliferative and secretory samples combined) endometrial epithelial cells unless stated otherwise.

Biological processes, functional classifications and gene annotations were analysed using Ingenuity Pathway Analysis software (IPA, Ingenuity Systems, Redwood City, CA, USA), DAVID (http://david.abcc.ncifcrf.gov) and GeneCards / Gene ALaCart (http://www.genecards.org). To identify biological processes with significant enrichment, the distribution of genes from our data were compared to a reference annotation gene list for each gene ontology category generated by IPA or DAVID. Fisher Exact P-value was used for gene enrichment analysis. The value ranges from 0 to 1 where Fisher Exact P-value equal to zero represents perfect enrichment. P-value $\leq$ 0.05 is considered significantly enriched in the annotation categories.

3.2.6 Transcriptional validation

Primers (Supplemental Table 3.1) were designed for candidate genes using primer bank database and 'primer 3' software. RNA was reverse-transcribed to cDNA using SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen) according to manufacturer's protocols. Quantitative RT-PCR was performed using SYBR Green PCR Master Mix and 7900 HT Fast Real-Time PCR System (Applied Biosystems). Each reaction was run in triplicate and consisted of 100 ng cDNA, 1-50 µM of optimised primers and 1X Fast Power SYBR Green mix. Amplification efficiency was determined using serially diluted endometrial epithelial cDNA (200 - 0.2 ng) using the slope of best fit curve for cycle threshold (Ct) and concentration. The amplification conditions were 95ºC

for 10 min, 95ºC for 15 sec and 60ºC for 1 min. No-template and no-RT controls were included for each assay to ensure quality and cDNA specificity of the primers. PCR products were verified by agarose gel electrophoresis. Target gene expression was normalised to 18S rRNA and relative gene expression assessed using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

3.2.7 Immunofluorescence

Fresh frozen full-thickness Pre-M and Post-M hysterectomy tissues were cryosectioned (5 µm), fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4, Invitrogen) for 20 min at room temperature (RT), permeabilised with 0.2% Triton X-100/PBS for 10 min, washed and incubated with protein blocking solution (Dako, CA, USA) for 10 min, followed by overnight incubation at 4ºC with primary antibodies; rabbit monoclonal anti human β-catenin (1:200, Abcam, Cambridge, UK) and rabbit polyclonal anti human Axin2 (1:100, Abcam). Sections were washed and incubated with AlexaFluor594 goat anti-rabbit IgG or AlexaFluor488 donkey anti-rabbit IgG (1:200, Molecular Probes, Oregon, USA) for 1 hour at RT. Negative controls were stained similarly but without primary antibody. Sections were washed and counterstained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen) for 15 min and mounted in fluorescence mounting medium (Dako). Images were captured and analysed using Leica DMR microscope and Leica IM50 v1.20 Image Manager (Leica Microsystems, Wetzlar, Germany) and Image J software (NIH, Bethesda, MD, USA).

3.2.8 Statistical analyses

Microarray data was analysed using RMA and ANOVA, and the gene list was filtered using $p < 0.05$ and fold change > 2. QRT-PCR validation data was analysed using Mann-Whitney U test (GraphPad Prism v5.01, CA, USA). Differences of $p < 0.05$ (95% confidence interval) were considered statistically significant.

3.3 Results

3.3.1 Differential gene expression between human Pre-M and Post-M endometrial epithelial cells

To determine whether the epithelial cells in thin, atrophic basalis-like Post-M endometrium expressed a different gene profile compared to the thick, highly proliferative functionalis of Pre-M (proliferative and secretory) endometrium, gene expression profiling of EpCAM magnetic bead-purified endometrial epithelial cells was conducted (Fig. 3.1). The transcriptional profile of 3 Post-M and 8 full-thickness Pre-M (4 proliferative and 4 secretory) endometrial epithelial samples identified 1,077 candidate genes with differential expression. Among these, 275 genes showed higher and 802 lower expression in Post-M compared to Pre-M (proliferative and secretory) endometrium.

To identify biological processes associated with these differentially expressed genes, we used DAVID to map the gene ontology (GO) classifications. Fisher Exact test was adopted to measure the gene-enrichment annotations. From a list of 275 highly expressed genes identified in Post-M, DAVID annotated 267 genes into 6 main categories including immune response, response to wounding, cell death regulation, negative regulation of cell proliferation, integrin mediated and Wnt signalling pathways (Table 3.2). From 802 lowly

expressed genes in Post-M, DAVID mapped 749 genes into 7 gene families including protein and intracellular transport, lipid biosynthesis process, protein kinase and tissue remodelling activity, cell cycle, cell migration and Wnt signalling pathway (Table 3.2). A complete gene ontology classification and gene list are provided as supplementary materials (Supplemental Tables 3.2-3.5 and Appendices 2-5).

TABLE 3.2 Gene ontology classifications of enriched biological processes in Post-M *vs.* Pre-M (proliferative and secretory) endometrial epithelial cells

Category	Fisher Exact P-value	Genes involved in category	Percentage (involved genes/total genes)
Post-M > Pre-M (267 genes)			
Immune response	2.80E-08	33	12.3
Response to wounding	2.40E-04	21	7.9
Cell death regulation	4.20E-03	22	8.2
Negative regulation of cell proliferation	4.40E-03	14	5.2
Integrin mediated signalling pathway	2.40E-02	5	1.9
Wnt signalling pathway	4.80E-02	8	3.0
Post-M < Pre-M (749 genes)			
Protein transport	1.70E-06	63	8.4
Intracellular transport	6.20E-06	55	7.3
Lipid biosynthetic process	3.70E-05	32	4.3
Protein kinase cascade	3.90E-05	35	4.7
Cell migration	5.50E-03	11	1.5
Cell cycle	4.80E-02	45	6.0
Wnt signalling pathway	6.80E-02	11	1.5

Fisher Exact P-value for the gene-enrichment category was generated from DAVID reference gene list. Genes involved in each category indicates the number of genes enriched for that category from the input gene list. Percentage indicates the relative number of involved genes from the total number of high or low expressing genes in individual categories. For example, the percentage of immune response is 33/267 = 12.3 %.

3.3.2 Wnt-associated signalling pathway

The Wnt signalling pathway was identified as a common GO biological process for endometrial epithelial cells (Table 3.2), confirming the importance of the Wnt pathway in endometrial development and function (Tulac *et al.*, 2003; Wang *et al.*, 2010). Further investigation using IPA software revealed a set of 22 Wnt-associated genes enriched in the differentially expressed gene set between Post-M and Pre-M endometrial epithelial cells (Fig. 3.2 and Supplemental Fig. 3.2). The dendrogram shows separate clustering of the Post-M and Pre-M samples for these Wnt-associated genes. For example, a similar differential gene expression pattern was observed between a late proliferative sample (sample 4) and a secretory (sample 5) compared to all 3 Post-M samples (samples 9-11) (Fig. 3.2). Of the 22 differentially expressed Wnt pathway genes, 14 showed increased and 8 decreased expression levels in Post-M compared to Pre-M (proliferative and secretory). Genes involved directly with the canonical Wnt pathway included a Wnt ligand (*WNT10A*), several receptors (*FZD2*, *FZD9*), a co-receptor (*LRP5*), negative regulators of the Wnt pathway (*AXIN2*, *GSK3β*) and downstream targets (*LEF1*, *MMP7*, *TLE1*, *CTBP2*). Other differentially expressed genes indirectly involved with the Wnt pathway were transcriptional factors (*SMO*, *SOX9*, *VANGL2*).

Given the distinct distribution and expression of Wnt signalling genes in epithelial cells in the proliferative and secretory phase of the menstrual cycle and the paucity of data on these genes in Post-M endometrial epithelial cells, the Wnt-associated genes were further analysed. We found 3 Wnt-associated genes were differentially expressed between Post-M and proliferative stage epithelial cells, 3 differentially expressed between Post-M and secretory stage and 9 were different between the proliferative and secretory stages of the menstrual cycle (Supplemental Table 3.2).

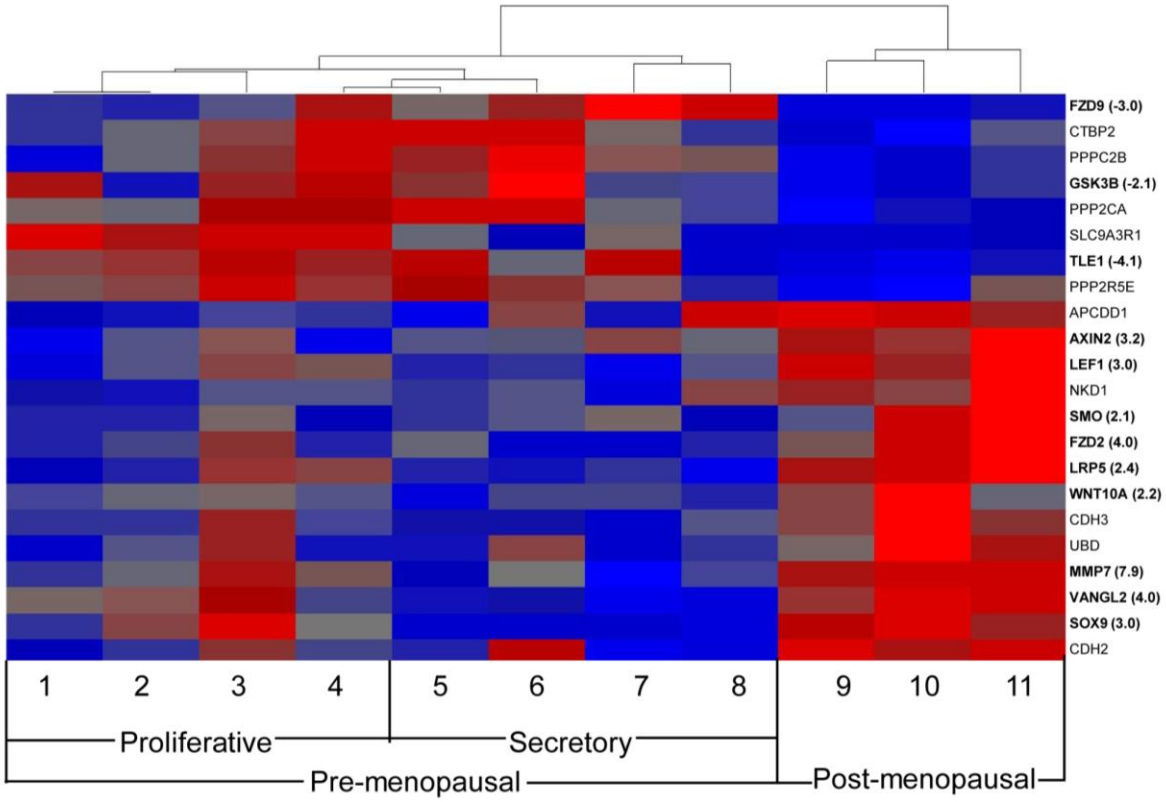


Figure 3.2 Differentially expressed genes involved in the Wnt signalling pathway in Pre-M and Post-M endometrial epithelial cells. Hierarchical cluster analysis of 22 Wnt-associated genes showing P < 0.05 (ANOVA) and a fold change of > 2 comparing proliferative (samples 1 – 4), secretory (samples 5–8) and post-menopausal (samples 9–11) endometrial epithelial cells. Each column shows the relative gene expression of a single patient sample for the Wnt-associated genes which are labelled on the abscissa. Higher expression (red) and lower expression (blue) is indicated by colour and intensity. qRT-PCR validation of selected genes and fold change are identified by bold lettering. Dendrogram and heat map were generated by Partek Genomics Suite software.

3.3.3 Validation of Wnt-associated genes

Nine genes of the canonical Wnt pathway and 3 transcription factors were validated by qRT-PCR (Fig. 3.3). *AXIN2, FZD2, MMP7, LEF1, LRP5, SMO, SOX9 VANGL2* and *WNT10A* were significantly increased in epithelial cells of Post-M compared to Pre-M (proliferative and secretory) endometrium. *FZD9, GSK3β* and *TLE1* had significantly lower expression. This validation was concordant with the gene profile data using RNA

both from the original microarray samples and from an extra set of patient samples (Table 3.1). This showed the sensitivity of identifying the differentially expressed genes between Post-M and Pre-M (proliferative and secretory) endometrium.

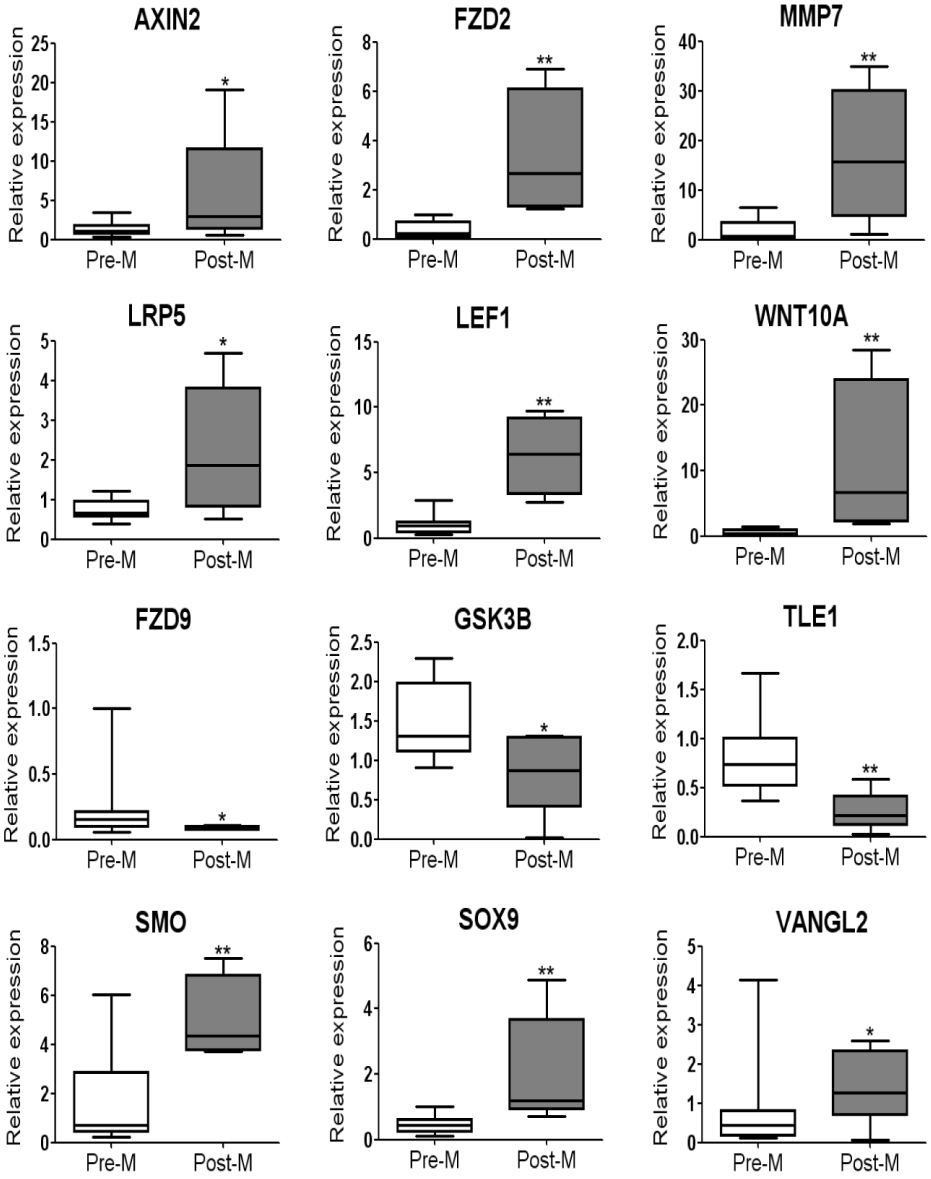


Figure 3.3 Validation of selected Wnt canonical pathway and transcription factor genes by qRT-PCR. All 12 genes examined showed statistically significant differences between Pre-M (proliferative and secretory, n = 14, white bar) and Post-M (n = 5, grey bar). Relative expression was normalised to 18S. Data are presented as whisker plots showing medians and minimum and maximum range (95% confidence interval). **P < 0.001; * P < 0.05.

3.3.4 Cellular localisation of key regulators of the Wnt pathway

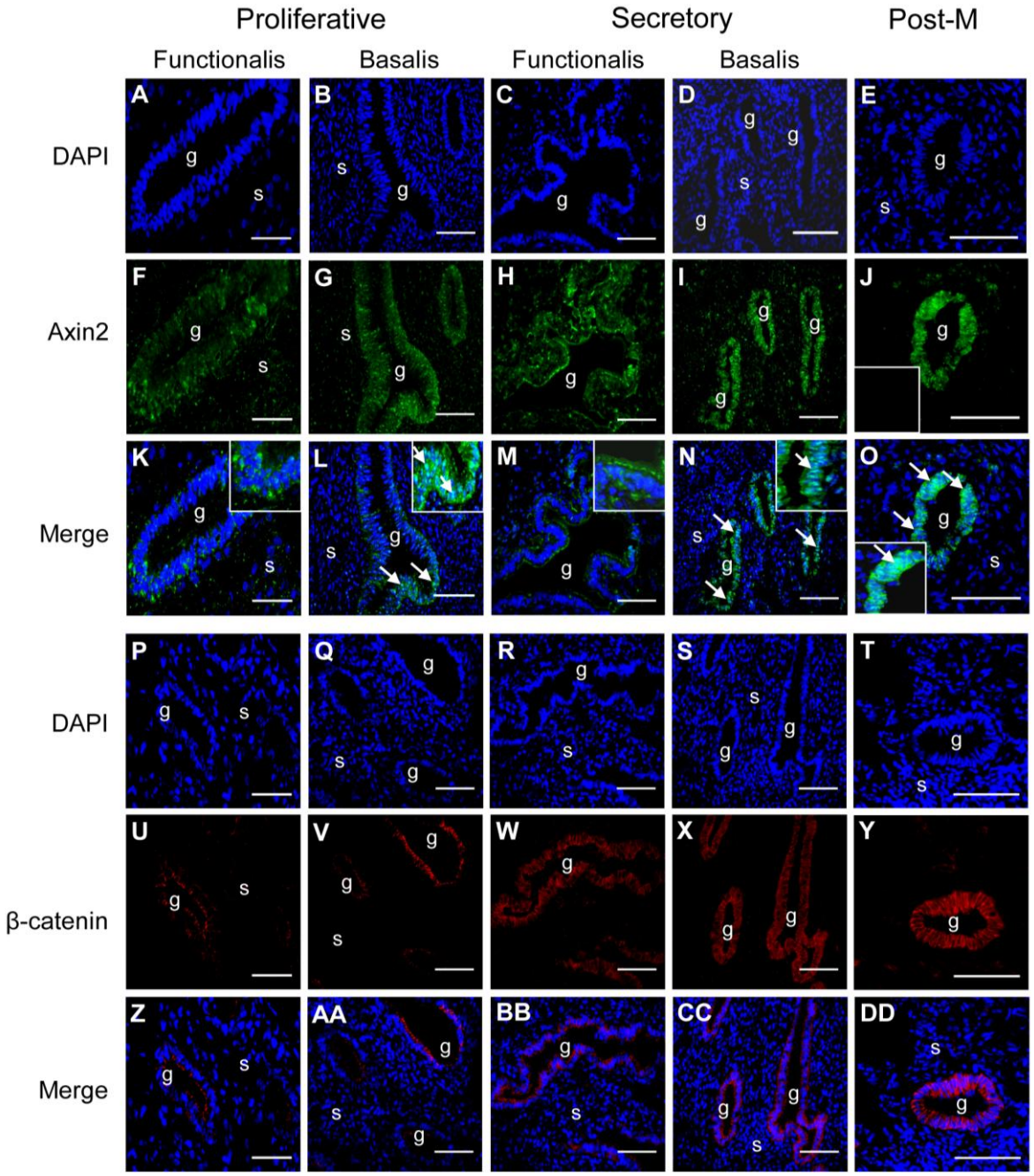
Axin2 localisation

Since Axin2 is a key negative regulator of β-catenin in the canonical Wnt / β-catenin pathway (Jho *et al.*, 2002; Cong & Varmus, 2004), the location of both Axin2 and β-catenin in full-thickness Pre-M and Post-M endometrium was investigated. Immunofluorescence revealed cytoplasmic staining of Axin2 in the functionalis of proliferative (Fig. 3.4F and K) and secretory endometrial glandular epithelia (Fig. 3.4H and M). In contrast, nuclear Axin2 staining was observed in the basalis of proliferative (Fig. 3.4G and L) and secretory Pre-M (Fig. 3.4I and N) and in Post-M endometrial epithelia (Fig. 3.4J and O).

β-catenin localisation

β-catenin was located in the cytoplasm and on the plasma membrane but not the nucleus of both functionalis (Fig. 3.4U, W, Z and BB) and basalis (Fig. 3.4V, X, AA and CC) of proliferative and secretory Pre-M, and Post-M endometrial glandular epithelium (Fig. 3.4Y and DD). β-catenin staining was more prominent in Post-M endometrium.

Figure 3.4 Immunofluorescence images of Axin2 and β-catenin in Pre-M and Post-M endometrium. Nuclear staining is shown with DAPI (A-E and P-T). Localisation of Axin2 in the functionalis (F and H) and basalis (G and I) glandular epithelium of proliferative (F,K), secretory (I, N) Pre-M and in Post-M (J, O) endometrium. Co-localisation of DAPI and Axin2 shows cytoplasmic staining in the functionalis (K and M) and nuclear staining in the basalis epithelium (L and N) of proliferative and secretory, and Post-M endometrium (O). Insets, Axin2 negative control (J), enlargement of Axin2 cytoplasmic localisation (K, M) in the functionalis and nuclear staining in basalis of Pre-M (L, N) and in Post-M endometrium (O). Arrows, Axin2 nuclear staining.
Localisation of cytoplasmic β-catenin in the functionalis (U, W, Z, BB) and basalis (V, X, AA, CC) glandular epithelium of proliferative and secretory Pre-M and in Post-M (Y, DD) endometrium. Co-localisation of DAPI and β-catenin shows cytoplasmic and plasma membrane staining in the functionalis (Z, BB), basalis (AA, CC) of proliferative and secretory and Post-M (DD) endometrium. g, glands; s, stroma. All images were taken at 40x magnification and are representative of 5 proliferative, 4 secretory and 3 post-menopausal endometrial samples.


Proliferative
Secretory
Post-M
Functionalis
Basalis
Functionalis
Basalis
DAPI
Axin2
Merge
DAPI
β-catenin
Merge
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
Y
Z
AA
BB
CC
DD
g
s

3.3.5 Enriched biological pathways

In this study, we also identified differentially expressed genes that are associated with stem cell signalling networks, stem cell regulation and cell fate determination, including *BMP (5 of 15), NOTCH (3 of 8), Hedgehog (3 of 7)* and *FGF* (7 of 19) signalling pathways (Table 3.3). To determine whether stem cell networks were menstrual cycle dependent, the data from Pre-M samples separated into proliferative and secretory, were compared with each other and individually with Post-M (Supplemental Table 3.3). This comparison showed 28 differentially expressed genes between Post-M and proliferative endometrial epithelial cells distributed across the *BMP (4), NOTCH (6), Hedgehog (*4) and *FGF (*14) signalling pathways (Supplemental Table 3.3). It showed 19 differentially expressed genes between Post-M and secretory samples in the *BMP* (7), *NOTCH* (2), *Hedgehog* (3) and *FGF* (7) pathways but only 3 between proliferative and secretory Pre-M samples (1 *NOTCH* and 2 *FGF* genes) (Supplemental Table 3.3). This finding highlights that the greatest number of significant differentially expressed stem cell network genes was between Post-M and Pre-M proliferative, and Post-M and Pre-M secretory epithelial cells.

A set of 9 genes involved in *endometrial cancer progression* were differentially expressed. This included 4 differentially expressed genes between Post-M and proliferative, 4 between Post-M and secretory and 3 between proliferative and secretory (Supplemental Fig. 3.3). Among these, several genes were associated with the Wnt signalling pathway, suggesting that Wnt pathway is important in endometrial development, function and proliferative diseases.

TABLE 3.3 Biological pathways associated with stem cell networks in the differentially expressed genes of human Post-M *vs.* Pre-M endometrial epithelial cells (P < 0.05)

Gene symbol	Gene description	Post-M *vs.* Pre-M	
		P-value (< 0.05)	Fold change
BMP signalling pathway			
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	0.028	-2.1
PRKAG2	protein kinase, AMP-activated, gamma 2 non-catalytic subunit	0.005	-2.5
MAPK13	mitogen-activated protein kinase 13	0.014	-2.9
BMP6	bone morphogenetic protein 6	0.026	-3.2
MAP2K1	mitogen-activated protein kinase kinase 1	0.010	-3.3
NOTCH signalling pathway			
DTX3	deltex homolog 3 (Drosophila)	0.001	3.5
NOTCH4	notch 4	0.017	2.3
HEY1	hairy/enhancer-of-split related with YRPW motif 1	0.013	-3.9
Hedgehog signalling pathway			
SMO	smoothened homolog (Drosophila)	0.015	2.1
DYRK1A	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A	0.030	-2.0
PRKAG2	protein kinase, AMP-activated, gamma 2 non-catalytic subunit	0.005	-2.5
FGF signalling pathway			
FGF9	fibroblast growth factor 9 (glia-activating factor)	0.015	4.7
FGF18	fibroblast growth factor 18	0.001	2.6
CRK	v-crk sarcoma virus CT10 oncogene homolog (avian)	0.018	-2.3
PIK3C2A	phosphoinositide-3-kinase, class 2, alpha polypeptide	0.040	-2.4
MAP2K3	mitogen-activated protein kinase kinase 3	0.002	-2.7
MAP2K1	mitogen-activated protein kinase kinase 1	0.010	-3.3
RPS6KA5	ribosomal protein S6 kinase, 90kDa, polypeptide 5	0.006	-3.5
Stem cell fate determination			
SOX9	SRY (sex determining region Y)-box 9	0.004	3.0
DACH1	dachshund homolog 1 (Drosophila)	0.006	2.4
KLF5	Kruppel-like factor 5 (intestinal)	0.031	-2.1
Stem cell communication regulators			
ASCL2	achaete-scute complex homolog 2 (Drosophila)	0.036	2.2
GJB1	gap junction protein, beta 1, 32kDa	0.023	-3.2
PPARG	peroxisome proliferator-activated receptor gamma	0.005	-6.0
Pluripotency maintenance and regulation			
IL6ST	interleukin 6 signal transducer (gp130, oncostatin M receptor)	0.004	-2.8
EDNRB	endothelin receptor type B	0.021	-3.5
SPHK1	sphingosine kinase 1	0.000	-8.1

Fold change are for comparison between Post-M *vs.* Pre-M endometrial epithelial cells. Positive values indicate high and negative values indicate low expression. Gene annotations were obtained from Gene ALaCart.

3.3.6 Other enriched gene families in endometrial epithelial cells

Genes of the GO category *negative regulation of cell proliferation* (indicative of cell quiescence) were enriched in Post-M endometrial epithelial cells, suggestive of minimal epithelial cell proliferation, including the putative ESP population, in Post-M endometrium. These genes included *RARRES1* (12.9 fold) and *RXRA* (2.6 fold), *DMBT1* (9.5 fold), *CEBPA* (2.7 fold) and *ADAMST1* (2.6 fold) (Table 3.4). *Immune/wounding response* genes including *S100A8* (11.8 fold), *S100A9* (11.4 fold) and *WDFC2* (2.6 fold) were also higher in Post-M compared to Pre-M endometrial epithelium (Table 3.4).

As expected, *cell cycle regulatory genes CDK7* (-2.2 fold), *CCNE1* (-2.3 fold), *WEE1* (-2.3 fold), *CDC5L* (-2.4 fold) and *CABLES1* (-5.2 fold) and *lipid/steroid hormone molecules, HMGCS1* (-2.8 fold), *HMGCR* (-3.6 fold), *PPARG* (-6.0 fold) and *HSD17B2* (-26.9 fold) were decreased in Post-M compared to Pre-M endometrial epithelium (Table 3.4), indicating the lack of epithelial growth in the absence of endogenous circulating estrogen. It also confirmed that Pre-M endometrial epithelium is modulated by hormones during the transition from estrogen stimulated proliferation to progesterone induced differentiation (Stagey *et al.*, 1991; Niklaus & Pollard, 2006).

Transporter molecules and members of the *secretoglobin family, SCGB2A2* (-6.0 fold), S*CGB1D2* (-31.1 fold) and *SCG1D4* (-90.1 fold) and the *metallothionein family, MT1E* (-2.4 fold), *MTE* (-2.7 fold), *MT1F* (-3.4 fold), *MTH* (-7.0 fold) and *MT1G* (-7.6 fold) were down-regulated in Post-M compared to Pre-M (proliferative and secretory) endometrial epithelial cells. *Tissue remodelling molecules* including members of the *matrix metalloproteinase family, MMP7* (7.9 fold), *MMP11* (4.6 fold) *and MMP25* (-2.4 fold) and the *MAPK kinase family* (fold change from -3.3 to -2.1) were also differentially expressed

between Post-M and Pre-M (proliferative and secretory) endometrial epithelial cells. This confirmed the role of MMPs in the monthly tissue remodelling of the endometrium and was consistent with earlier studies (Kao *et al.*, 2002; Gaide Chevronnay *et al.*, 2010). In addition, the *keratin family, KRT17* (7.8 fold), *KRT18* (-2.1 fold), *KRTA4-7,* (-2.2 fold), *KRT19* (-2.5 fold), *KRT80* (-2.8 fold) and *KRT13* (-3.5 fold) also showed differential expression between Post-M and Pre-M (proliferative and secretory), suggesting possible novel epithelial-specific markers that could distinguish between epithelium in the functionalis and basalis endometrium.

3.3.7 Differentially regulated endometrial epithelial genes in common with other published literature

We propose that Post-M endometrium is similar to the basalis of Pre-M endometrium and that an ESP cell population resides in the basalis. However, the gene profile of epithelial cells in Post-M and the basalis of Pre-M endometrium has not been previously examined. We therefore compared our gene set to published microarray datasets on endometrial epithelial cells isolated from functionalis *versus* basalis using laser capture microdissection (LCM) (Gaide Chevronnay *et al.*, 2009; Gaide Chevronnay *et al.*, 2010). From a list of 71 genes identified in the Gaide Chevronnay et al study (Gaide Chevronnay *et al.*, 2009) comparing epithelial cells from functionalis and basalis layer, 21 genes were found in common with Post-M and Pre-M epithelial samples of this study (Supplemental Table 3.4). Seven of the 21 genes showed increased and 14 genes decreased expression in Post-M compared with Pre-M (proliferative and secretory). This represents a 6.5 fold enrichment of Pre-M basalis genes in Post-M epithelium than would be expected for random distribution.

In another comparative analysis from a list of 416 differentially expressed genes between endometrial explants cultured with and without sex steroid hormones (Gaide Chevronnay *et al.*, 2010), a set of 57 genes were found in common with the present study comparing uncultured Post-M and Pre-M (proliferative and secretory) endometrial epithelial cells. Of these, 12 genes showed increased and 45 decreased expression in Post-M compared to Pre-M respectively (Supplemental Table 3.5).

TABLE 3.4 List of highly enriched gene families differentially expressed between Post-M and Pre-M endometrial epithelial cells (P < 0.05 and fold change >2)

Gene symbol	Gene description	Post-M *vs.* Pre-M	
		P-value (< 0.05)	Fold change
Regulation of cell proliferation			
RARRES1	retinoic acid receptor responder (tazarotene induced) 1	0.005	12.90
DMBT1	deleted in malignant brain tumors 1	0.031	9.48
VANGL2	vang-like 2 (van gogh, Drosophila)	0.002	3.96
SOX9	SRY (sex determining region Y)-box 9	0.004	2.99
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha	0.001	2.77
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	0.006	2.64
RXRA	retinoid X receptor, alpha	0.030	2.61
SMO	smoothened homolog (Drosophila)	0.015	2.06
Defence response			
S100A9	S100 calcium binding protein A9	0.027	11.83
S100A8	S100 calcium binding protein A8	0.003	11.43
WFDC2	WAP four-disulfide core domain 2	0.001	2.65
CCL4L1	chemokine (C-C motif) ligand 4-like 1	0.031	2.45
Cell cycle regulators			
CDKN1C	cyclin-dependent kinase inhibitor 1C	0.018	2.00
CDK7	cyclin-dependent kinase	0.025	-2.20
CCNE1	cyclin E1	0.024	-2.30
WEE1	WEE1 homolog (S. pombe)	0.014	-2.33
CDC5L	cell division cycle 5-like	0.015	-2.40
CABLES1	Cdk5 and Abl enzyme substrate 1	0.000	-5.16
Lipid biosynthesis process			
CH25H	cholesterol 25-hydroxylase	0.000	4.67
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)	0.011	-2.80
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase	0.015	-3.64
PPARG	peroxisome proliferator-activated receptor gamma	0.005	-6.00
HSD17B2	hydroxysteroid (17-beta) dehydrogenase 2	0.001	-26.88
Secretoglobin family			
SCGB3A1	secretoglobin, family 3A, member 1	0.002	6.19
SCGB2A2	secretoglobin, family 2A, member 2	0.040	-6.00
SCGB1D2	secretoglobin, family 1D, member 2	0.005	-31.11
SCGB1D4	secretoglobin, family 1D, member 4	0.002	-90.07
Metallothionein family			
MT1E	metallothionein 1E	0.015	-2.40
MTE	metallothionein 1I (pseudogene)	0.028	-2.73
MT1F	metallothionein 1F	0.003	-3.38
MT1H	metallothionein 1H	0.000	-6.95
MT1G	metallothionein 1G	0.001	-7.57

Table 3.4 continued

Gene symbol	Gene description	Post-M *vs.* Pre-M	
		P-value (< 0.05)	**Fold change**
Extracellular matrix, tissue remodelling			
MMP7	matrix metallopeptidase 7 (matrilysin, uterine)	0.015	7.91
MMP11	matrix metallopeptidase 11 (stromelysin 3)	0.030	4.65
MMP25	matrix metallopeptidase 25	0.045	-2.39
MAP4K5	mitogen-activated protein kinase kinase kinase kinase 5	0.016	-2.11
MAP4K2	mitogen-activated protein kinase kinase kinase kinase 2	0.003	-2.12
MAP2K3	mitogen-activated protein kinase kinase 3	0.002	-2.71
MAP4K3	mitogen-activated protein kinase kinase kinase kinase 3	0.043	-2.79
MAPK13	mitogen-activated protein kinase 13	0.014	-2.94
MAP2K1	mitogen-activated protein kinase kinase 1	0.010	-3.27
Keratin family			
KRT17	keratin 17	0.015	7.80
KRT18	keratin 18	0.042	-2.10
KRTAP4-7	keratin associated protein 4-7	0.045	-2.17
KRT19	keratin 19	0.016	-2.49
KRT80	keratin 80	0.025	-2.82
KRT13	keratin 13	0.004	-3.53

Fold change for each gene is shown; positive values indicate higher and negative values indicate lower expression between Post-M and Pre-M (proliferative and secretory) endometrial epithelial cells. Data was generated from IPA and DAVID databases. Gene annotations were obtained from Gene ALaCart.

3.4 Discussion

In this study, we compared gene expression profiles of purified epithelial cells isolated from full-thickness Pre-M (proliferative and secretory) and basalis-like Post-M endometrium to identify gene pathways that distinguish the quiescent Pre-M basalis and Post-M epithelium from the rapid remodelling functionalis epithelium. Hierarchical clustering analyses revealed major gene differences between Post-M and Pre-M endometrial epithelial cells, and in particular 22 Wnt-associated genes were modulated in Post-M compared to Pre-M (proliferative and secretory) endometrial epithelial cells. These genes included a Wnt ligand (*WNT10A*), Wnt receptors (*FZD2, FZD9*) and co-receptor (*LRP5*), negative regulators of the Wnt pathway (*AXIN2, GSK3β*) and downstream transcriptional factor/targets of the Wnt pathway (*MMP7*, *LEF1*, *TLE1*, *CTBP2)*. The differential expression of several members of the canonical Wnt pathway within the endometrial epithelial compartment was striking, especially in Post-M, suggesting that Wnt signalling is likely down-regulated in Post-M endometrium, owing to the nuclear location of the negative regulator, Axin2. This would explain the quiescent state of Post-M endometrial epithelium until it is re-exposed to estrogen (Hou *et al.*, 2004; Klaassens *et al.*, 2006). Furthermore, components of the Wnt cascade are hormonally regulated as a large number of genes were differentially expressed between cycling (Pre-M) and non-cycling (Post-M) endometrium, confirming and extending previous studies in mouse and human endometrium (Bui *et al.*, 1997; Miller *et al.*, 1998). These findings suggest that the Wnt signalling pathway is important in endometrial epithelial growth and regression, possibly through down-regulating the proliferative activity of ESP cells postulated to reside in the basalis. In addition, comparison of our data with that of Gaide Cheveronnay (Gaide Chevronnay *et al.*, 2009; Gaide Chevronnay *et al.*, 2010) demonstrates and confirms that

Post-M endometrial epithelium has a similar gene expression profile to the basalis of Pre-M endometrium.

Our identification of cytoplasmic β-catenin expression in glandular epithelial cells of Post-M and proliferative and secretory stages of Pre-M endometrium is consistent with previous reports (Tulac *et al.*, 2003; Hou *et al.*, 2004). Nuclear location of Axin2 but not β-catenin was observed in our study, suggesting that Axin2 may work with Gsk3β to retain β-catenin in the proteosomal degradation complex (Caspi *et al.*, 2008; Cadigan & Peifer, 2009). More importantly, nuclear staining of Axin2 in Post-M endometrial epithelium indicates negative feedback action on the canonical Wnt pathway to maintain cytoplasmic β-catenin and subsequent down regulation of downstream transcriptional targets (Jho *et al.*, 2002; Leung *et al.*, 2002; Cong & Varmus, 2004). Nuclear Axin2 was selectively found in the basalis of proliferative and secretory Pre-M endometrial epithelial cells, indicating the similarity in Wnt pathway activity between the basalis of Pre-M and Post-M endometrium. Hierarchical clustering showed similar gene expression patterns between proliferative and secretory epithelium in comparison to Post-M endometrial epithelium. Together these findings suggest that Axin2 regulates Wnt signalling (Jho *et al.*, 2002; Cong & Varmus, 2004) to maintain the relative quiescent state of Post-M glandular epithelium. Wnt signalling is essential for maintaining stem/progenitor cell function, particularly in the intestine, where it is involved in stem cell self-renewal and Paneth cell differentiation (van de Wetering *et al.*, 2002). Similarly, Wnt signalling is involved in endometrial growth and differentiation during the menstrual cycle (Nei *et al.*, 1999; Wang *et al.*, 2010), thus we suggest that it may have a role in regulating the endometrial ESP cell compartment.

The differential expression and enrichment of *BMP, NOTCH, Hedgehog and FGF* signalling pathways genes identified in this study suggests the possible existence of stem cell signalling networks. More stem cell pathway genes were identified when Pre-M samples were separated into proliferative and secretory and individually compared to Post-M (Supplemental Table 3.3), emphasising the importance of stem cell signalling pathways in Post-M epithelium. The co-existence of Wnt signalling molecules and *BMP, NOTCH, Hedgehog and FGF* signalling cascades suggests crosstalk between these pathways which may function to regulate adult stem cell fate (Katoh, 2007; Nakamura *et al.*, 2007; Shin *et al.*, 2011). It is possible that crosstalk between Wnt and these developmental pathways may have a role in regulating and maintaining ESP cells to control epithelial homeostasis, further strengthening the hypothesis that this population may be located within the basalis layer of the endometrium. However, it is important to acknowledge that the ESP cell compartment is very small compared to the remaining epithelial cells, even in Post-M endometrium, and that these stem cell signalling pathways may have roles in "basalis-like" epithelial cells.

It is of interest that *negative regulation of cell proliferation* (indicates quiescence) genes were highly expressed in basalis-like Post-M endometrial epithelium. Highly prominent genes in this category were the *cell proliferation regulator, RXRA*, whose expression was expected in atrophic glands (Alfaro *et al.*, 2003; Wu *et al.*, 2004), a *tissue remodelling gene ADAMST1* and an *estrogen modulated gene, DMBT1,* essential for epithelial proliferation and regeneration (Tan *et al.*, 2003; Ace & Okulicz, 2004). The annotation of these highly expressed genes in Post-M endometrium suggests their possible functional role in regulating endometrial epithelial cell proliferation in the basalis of Pre-M and their potential roles in regulating putative resident ESP cells. This study also found that c*ell*

cycle regulatory genes had lower expression in Post-M compared to Pre-M endometrial epithelium, confirming previous reports showing the slow proliferation rate and reduced mitotic activity of Post-M and basalis of Pre-M endometrium (Ferenczy *et al.*, 1979; Padykula *et al.*, 1989; Felix & Farahmand, 1997; Gargett, 2007).

HSD17β2, a key target of estrogen metabolism and regulated by progesterone, was highly expressed in Pre-M (proliferative and secretory), consistent with previous reports (Mustonen *et al.*, 1998; Tan *et al.*, 2003; Talbi *et al.*, 2006). The low expression level of *HSD17β2* in basalis-like Post-M endometrium reflects the low estrogen hormonal status (Gargett, 2007; Kurman *et al.*, 2011). The *extracellular matrix/tissue remodelling* genes found in the present study agree with others (Rodgers *et al.*, 1994; Carson *et al.*, 2002; Gaide Chevronnay *et al.*, 2010) indicating the active involvement of these molecules in remodelling the endometrium throughout a women's reproductive years (Gaide Chevronnay *et al.*, 2010). The diminished expression of *transporter molecules* and the *secretoglobin family* in Post-M compared to Pre-M endometrium (Muller-Schottle *et al.*, 1999; Kao *et al.*, 2002; Ace & Okulicz, 2004) indicates the quiescent state of Post-M endometrial epithelium and confirms the high level of cellular nutrient uptake in cycling functionalis endometrium and their important role in endometrial receptivity. Similarly, members of the *metallothionein family* found lowly expressed in Post-M compared with Pre-M (proliferative and secretory) endometrial epithelial cells, suggest their important role in detoxification and protection of proliferating cells from heavy metal toxicity in the latter (Ioachim *et al.*, 2000; Kao *et al.*, 2002). Members of the *keratin family* identified in this study could be useful to distinguish basalis and functionalis epithelial cells in cycling and non-cycling endometrium. Further characterisation of these members is required to determine their specific role in endometrial epithelium.

In this study, the differential gene expression data generated from human endometrial epithelial cells was concordant with a LCM gene array study comparing basalis and functionalis epithelium in menstruating endometrium (Gaide Chevronnay *et al.*, 2009; Gaide Chevronnay *et al.*, 2010). Our comparative analysis showed that differentially expressed genes obtained from Post-M samples were similar to gene profiles of epithelial cells in the basalis layer of menstrual Pre-M endometrium (Gaide Chevronnay *et al.*, 2009). Furthermore, highly expressed genes in Post-M endometrial epithelial cells identified in our study were found in common and similar with gene profiles of endometrial glands in explants cultured in the absence of hormones (Gaide Chevronnay *et al.*, 2010). These two key comparisons between our study and that of Gaide Chevronnay (Gaide Chevronnay *et al.*, 2009; Gaide Chevronnay *et al.*, 2010) further support our proposal that Post-M is similar to the basalis of Pre-M endometrium, and that it operates independently of sex steroid hormones (Kurman *et al.*, 2011). The few differences in gene expression observed between the comparative studies could be due to different experimental design, method of epithelial cell isolation and microarray platform used.

To our knowledge, the gene expression data presented here is the first to analyse and compare purified epithelial cells isolated from Pre-M and Post-M endometrium of hysterectomy tissue using highly specific magnetic bead sorting. The identification of endometrial epithelial-specific genes *(MMP7, WNT7A*) and the notable absence of stromal-specific genes (*WNT4, DKK*) validates our approach. These epithelial-specific genes are consistent with previous reports (Tulac *et al.*, 2003; Gaide Chevronnay *et al.*, 2009; Gaide Chevronnay *et al.*, 2010), indicating that our technique used for isolating homogenous human endometrial epithelial cells was highly selective. Our experimental design comparing full-thickness, hormone responsive cycling Pre-M endometrium to basalis-like,

hormone-depleted Post-M endometrium has elucidated a likely gene profile for basalis endometrial epithelium which would normally be masked by genes contributed by the more active and abundant functionalis layer. We also reasoned that the ESP population would be enriched in Post-M samples as there was no functionalis to dilute their contribution to the gene expression as in Pre-M samples. The identification of many differentially expressed stem cell network genes between Post-M and Pre-M (proliferative and secretory), including the 22 Wnt signalling pathway genes supports this contention. Similarly, given the relatively small contribution of the basalis in Pre-M samples, the gene expression signal for any given gene is diluted by this proportion. It is expected that a slight diminution in fold change is relative to the proportion of basalis compared to the functionalis of Pre-M samples. However, the main variable for obtaining statistical significance for differential expression is consistency between samples.

This study lays the groundwork for several immediate investigations including the identification of endometrial ESP cell markers and examining the role of ESP cells in endometrial epithelial regeneration. Previous studies have shown that regeneration of the functionalis epithelium originates from remaining gland stumps located in the basalis (Ferenczy, 1976; Ludwig & Metzger, 1976). Rare clonogenic epithelial cells likely have a role (Chan *et al.*, 2004; Schwab *et al.*, 2005), however, the exact origin and location of endometrial ESP cells remain unknown. Several morphological studies suggest that regeneration of new endometrial epithelium during menses results from stromal differentiation (Garry *et al.*, 2009, 2010). Alternatively, vascular endothelial growth factor may have a role as it is important for luminal epithelium migration during repair and endometrial regeneration in primate and mouse models (Fan *et al.*, 2008). We suggest that basalis gene products contribute to endometrial regeneration via regulating the ESP cell

compartment while functionalis gene products may be involved in amplifying the stem cell response, differentiation and endometrial breakdown (Chan & Gargett, 2006; Gargett, 2007; Chan *et al.*, 2011). The data presented in this study could be used to extend previous reports on endometrial regeneration (Fan *et al.*, 2008; Garry *et al.*, 2009, 2010) to elucidate the origin and location of ESP cells. The involvement of epithelial-specific genes in hormone-induced regeneration could be broadened by comparing this study to that of previous published gene expression profiles of Post-M endometrium treated with hormones (Klaassens *et al.*, 2006; Hanifi-Moghaddam *et al.*, 2007).

This study comparing the transcriptome of basalis-like Post-M and full-thickness Pre-M endometrial epithelial cells provides evidence suggesting a similar molecular gene signature between Post-M and Pre-M basalis epithelium. However, the specific gene contribution from the luminal and glandular epithelium was not addressed. Future studies using specific markers distinguishing luminal and glandular epithelium are needed to determine the relative roles of these two epithelial compartments in endometrial epithelial regeneration. Our quantitative data was consistent for all samples examined, as the underlying non-endometrial pathologies of prolapse, fibroids and menorrhagia showed no apparent effect, and adenomyosis samples were only used for immunohistochemical analysis.

An important finding of this study is that an intact canonical Wnt signalling cascade is present within the endometrial epithelial compartment in both Pre-M and Post-M endometrium. At the transcript level, these Wnt molecules are differentially expressed between Post-M and Pre-M endometrial epithelial cells. At a protein level, we have shown that Axin2, a key negative regulator of the canonical Wnt signalling pathway likely

shuttles between cytoplasmic and nuclear compartments, suggesting that Axin2 is actively involved in β-catenin regulation in human endometrium. This finding indicates that the Wnt signalling pathway is important in endometrial epithelial function, especially in Post-M and in the basalis of Pre-M endometrium where there is minimum proliferative activity. The Wnt signalling pathway may influence endometrial epithelial regeneration through regulating cell fate decisions of the ESP cell population possibly present in the basalis through interactions with niche cells. Given the essential role of Wnt signalling in stem cell biology, current investigations in our laboratory are extending the present finding, by identifying markers of endometrial ESP cells and examining the role of Wnt signalling in these cells.

3.5 Acknowledgements

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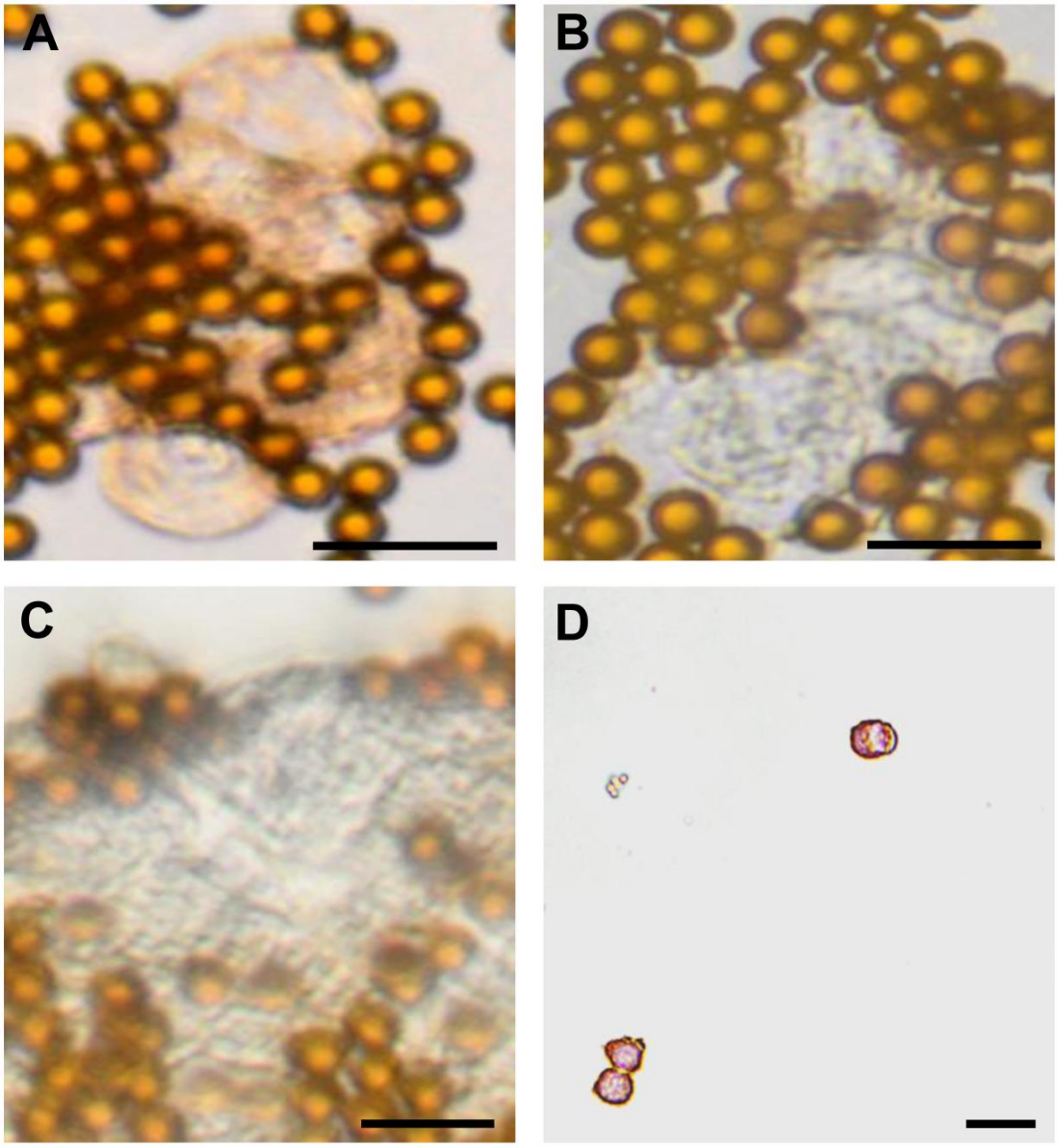
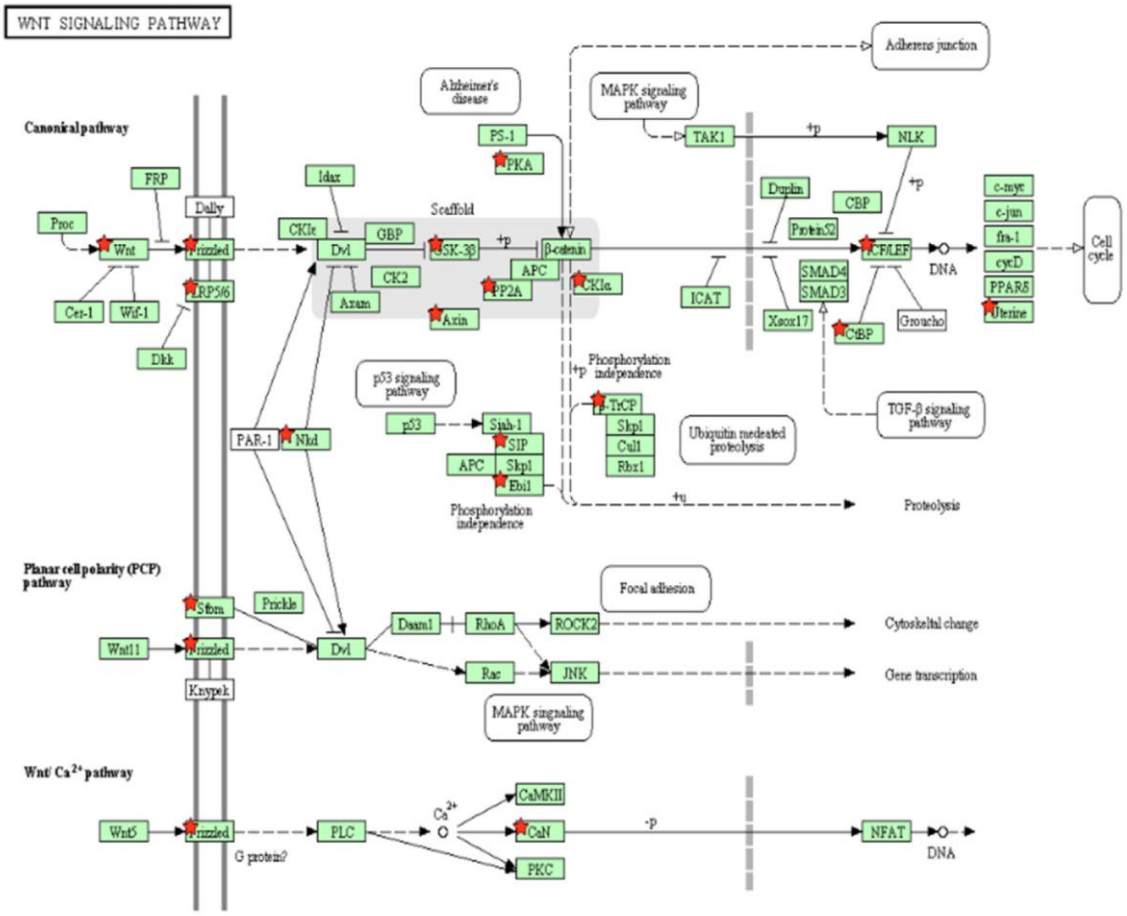
The authors declare that there are no conflicts of interest.

3.6 Supplementary Information

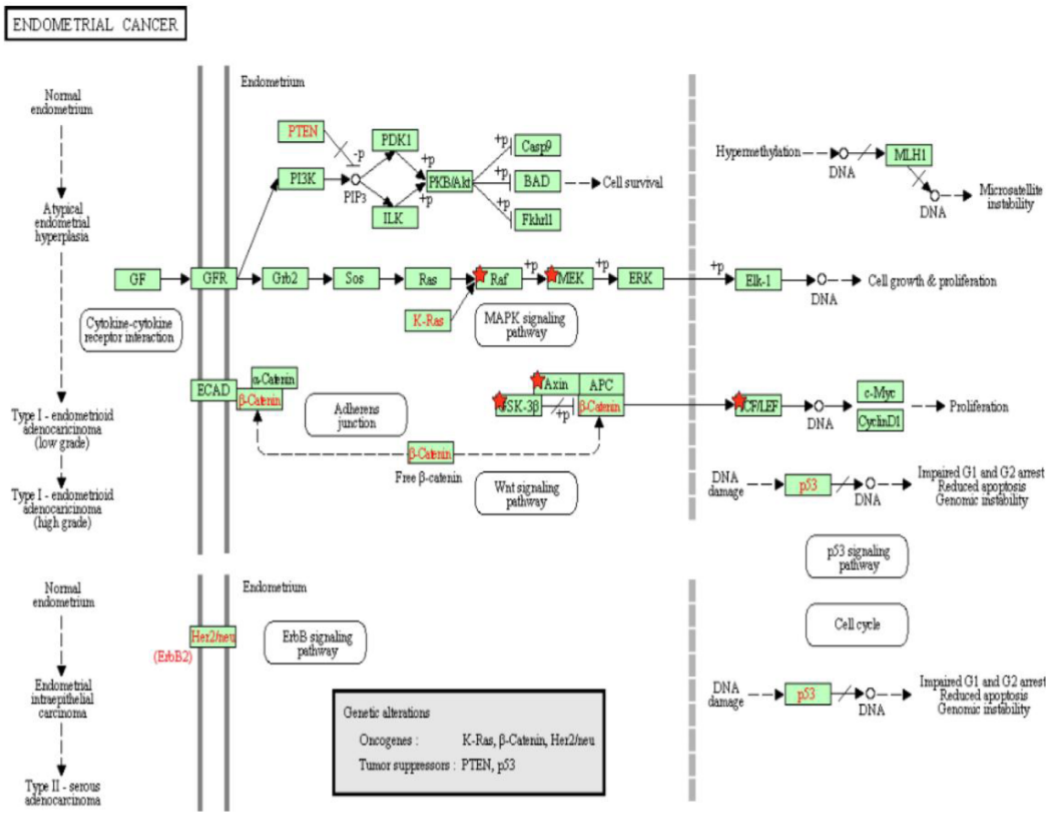


Supplemental Figure 3.1 Immunohistochemistry of bead-selected endometrial epithelial cells. Epithelial cells were surrounded by magnetic anti-EpCAM antibody-coated beads and were (A) positive for cytokeratin, (B) negative for stromal marker CD90. (C) Negative control, mIgG_1. (D) Positive control, stromal cells immunostained with CD90. Scale bars = 1µm.



Supplemental Figure 3.2 Differential expression of genes involved in the Wnt signalling pathway. This pathway was generated by DAVID and KEGG, each box represents a gene from the KEGG database. Gene boxes with a red star indicate differentially expressed genes identified in this study. Three different Wnt signalling pathways are shown, with 15 genes identified in the canonical Wnt pathway, 2 genes in the planar/cell polarity pathway and 2 genes in the Wnt / Ca^{2+} pathway.

A



B

Gene symbol	Gene description	Fold change		
		Post-M *vs.* P	Post-M *vs.* S	P *vs.* S
AXIN1	axin 1	-2.19		
CASP9	caspase 9, apoptosis-related cysteine peptidase			-2.10
CCND1	cyclin D1			2.61
FOXO3	forkhead box O3	-2.32		
GRB2	growth factor receptor-bound protein 2		-2.04	
LEF1	lymphoid enhancer-binding factor 1	2.67	3.46	
MAP2K1	mitogen-activated protein kinase kinase 1	-2.97	-3.59	
PIK3C2A	phosphoinositide-3-kinase, class 2, alpha polypeptide		-3.03	
PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (alpha)			-2.54

Supplemental Figure 3.3 Genes associated with endometrial cancer progression pathway. (A) Endometrial cancer progression pathway generated by DAVID and KEGG databases. Five differentially expressed genes (indicated by red star) between Post-M and Pre-M were also components of the Wnt pathway. (B) Additional list of genes associated with endometrial cancer progression differentially expressed between Post-M *vs.* proliferative (P), Post-M *vs.* secretory (S) and P *vs.* S.

Supplemental Table 3.1 List of primers used for qRT-PCR validation

Gene symbol	Forward primer (5' - 3')	Reverse primer (5' - 3')
AXIN2	gtgtgaggtccacggaaact	tggctggtgcaaagacatag
FZD2	aaggtgccatcctatctcagc	tagcagcccgacagaaaaatg
FZD9	gcggcaccaacacagagaa	agacatagcaaacgatgacgc
GSK3β	aactgcccgactaacaccac	attggtctgtccacggtctc
LEF1	aatgagagcgaatgtcgttgc	gctgtctttctttccgtgcta
LRP5	tcctgctatttgccaaccg	gactccagcttgactccgc
MMP7	ggaggagatgctcacttcgat	aggaatgtcccatacccaaaga
SOX9	agcgaacgcacatcaagac	gctgtagtgtgggaggttgaa
SMO	ctttgtcatcgtgtactacgcc	cgagagaggctggtaggtg
VANGL2	tggcaaaccctgatgatgta	gaggaacaggggagttagcc
TLE1	cagttcccttacgcctcac	atcgtggtgcttcttgtcatc
WNT10A	gccaacaccaattcagggac	gcgatggcgtaggcaaaag

Sequences are presented 5’ to 3’.

Supplemental Table 3.2 Additional differentially expressed Wnt signalling genes identified in Post-M *vs.* proliferative, Post-M *vs.* secretory and proliferative *vs.* secretory endometrial epithelial cells

Gene symbol	Gene description	Fold change		
		Post-M *vs.* P	Post-M *vs.* S	P *vs.* S
AXIN1	axin 1	-2.19		
SOX13	SRY (sex determining region Y)-box 13	-2.49		
TLE4	transducin-like enhancer of split 4 (E(sp1) homolog, Drosophila)	-2.86		
TCF3	transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)		2.43	
GNAQ	guanine nucleotide binding protein (G protein), q polypeptide		-2.57	
CD44	CD44 molecule (Indian blood group)		-3.30	
TGFB2	transforming growth factor, beta 2			-3.79
PPP2R5A	protein phosphatase 2, regulatory subunit B', alpha			-3.19
ACVR1B	activin A receptor, type IB			-2.35
RARA	retinoic acid receptor, alpha			2.46
CCND1	cyclin D1			2.61
SOX4	SRY (sex determining region Y)-box 14			2.70
BCL9	B-cell CLL/lymphoma 9			2.80
SFRP1	secreted frizzled-related protein 1			3.17
WNT7A	wingless-type MMTV integration site family, member 7A			4.01

Fold change for each individual gene is shown. This gene list was generated from IPA analysis of the microarray data generated in this study.

Supplemental Table 3.3 Differentially expressed stem cell signalling network genes identified in Post-M *vs.* proliferative, Post-M *vs.* secretory and proliferative *vs.* secretory endometrial epithelial cells

Gene symbol	Gene description	Fold change		
		Post-M *vs.* P	Post-M *vs.* S	P *vs.* S
BMP signalling pathway				
BMP6	bone morphogenetic protein 6		-4.61	
GRB2	growth factor receptor-bound protein 2		-2.04	
MAP2K1	mitogen-activated protein kinase kinase 1	-2.97	-3.59	
MAPK13	mitogen-activated protein kinase 13	-2.45	-3.52	
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	-2.45		
PRKAG2	protein kinase, AMP-activated, gamma 2 non-catalytic subunit	-2.08	-2.98	
PRKAR2A	protein kinase, cAMP-dependent, regulatory, type II, alpha		-2.08	
RELA	v-rel reticuloendotheliosis viral oncogene homolog A (avian)		-2.35	
NOTCH signalling pathway				
DLL1	delta-like 1 (Drosophila)	2.84		-2.69
DTX3	deltex homolog 3 (Drosophila)	3.35	3.62	
HES1	hairy and enhancer of split 1, (Drosophila)	2.78		
HEY1	hairy/enhancer-of-split related with YRPW motif 1	-6.16		
HES5	hairy and enhancer of split 5 (Drosophila)	-2.53		
NOTCH4	notch 4	2.08	2.49	
Hedgehog signalling pathway				
DYRK1A	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A	-2.04	-2.05	
PRKAG2	protein kinase, AMP-activated, gamma 2 non-catalytic subunit	-2.08	-2.08	
SMO	smoothened homolog (Drosophila)	2.12	2.01	
STK36	serine/threonine kinase 36	2.00		
FGF signalling pathway				
CRK	v-crk sarcoma virus CT10 oncogene homolog (avian)	-2.47	-2.11	
CREB5	cAMP responsive element binding protein 5	-2.35		-2.35
FGF9	fibroblast growth factor 9 (glia-activating factor)	2.39	9.36	
FGF18	fibroblast growth factor 18	2.54	2.61	
FGF19	fibroblast growth factor 19	3.39		
FGFR3	fibroblast growth factor receptor 3	4.57		
GRB2	growth factor receptor-bound protein 2	-2.04		
MAP2K1	mitogen-activated protein kinase kinase 1	-2.97	-3.59	
MAP2K3	mitogen-activated protein kinase kinase 3	-2.52	-2.92	

MAP3K5	mitogen-activated protein kinase kinase kinase 5	-4.26		
MAPK13	mitogen-activated protein kinase 13	-2.45	-3.52	-2.45
PIK3C2A	phosphoinositide-3-kinase, class 2, alpha polypeptide	-3.03		
PTPN11	protein tyrosine phosphatase, non-receptor type 11	-2.14		
RPS6KA5	ribosomal protein S6 kinase, 90kDa, polypeptide 5	-2.53	-4.92	

Supplemental Table 3.4 Comparative analysis of differentially expressed genes in common between this study and that of Gaide Chevronnay et. al. 2009, investigating endometrial remodelling

Gene symbol	Gene description	Observation		
		Fold change Post-M *vs.* Pre-M (our study)	Our study	Gaide Chevronnay et. al. 2009
i) Lysis functionalis > Basalis = Pre-M > Post-M				
VNN1	vanin 1	-9.47	Down	Down
TFPI2	tissue factor pathway inhibitor 2	-4.88	Down	Down
SFN	stratifin	-4.74	Down	Down
SLC38A1	solute carrier family 38, member 1	-3.48	Down	Down
MT1F	metallothionein 1F	-3.38	Down	Down
SPINK1	serine peptidase inhibitor, Kazal type 1	-3.24	Down	Down
SERPINB9	serpin peptidase inhibitor, clade B (ovalbumin), member 9	-2.98	Down	Down
TNFAIP3	tumor necrosis factor, alpha-induced protein 3	-2.84	Down	Down
IL8	interleukin 8	-2.51	Down	Down
MT1E	metallothionein 1E	-2.40	Down	Down
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	-2.37	Down	Down
ADM2	adrenomedullin 2	-2.31	Down	Down
SOD2	superoxide dismutase 2, mitochondrial	-2.15	Down	Down
DDIT4L	DNA-damage-inducible transcript 4-like	5.91	Up	Down
LCN2	lipocalin 2	10.68	Up	Down
HBB	hemoglobin, beta	12.72	Up	Down
ii) Lysis functionalis < Basalis = Pre-M < Post-M				
LAMA2	laminin, alpha 2	-4.15	Down	Up
DACH1	dachshund homolog 1 (Drosophila)	2.45	Up	Up
MATN2	matrilin 2	4.05	Up	Up
SCARA3	scavenger receptor class A, member 3	5.53	Up	Up
HLA-DPA1	major histocompatibility complex, class II, DP alpha 1	12.08	Up	Up

Differentially expressed genes between Pre-M and Post-M endometrial epithelium of this study are similar and found in common with that of laser capture microdissection of lysis functionalis and basalis epithelium respectively. Fold change shown here were generated by Partek software analysing microarray data of this study. Comparative observation for each gene between this study and Gaide Chevronnay et. al. 2009 is indicated.

Supplemental Table 3.5 Comparative analysis of genes in common between this study and that of Gaide Chevronnay et. al. 2010, investigating glands of explants treated with/without hormones

Gene symbol	Gene description	Observation		
		Fold change Post-M *vs.* Pre-M (our study)	Our study	Gaide Chevronnay et. al. 2010
i) -H > +H equivalent to Post-M > Pre-M				
HLA-DRA	major histocompatibility complex, class II, DR alpha	8.63	Up	Up
HLA-DRB1	major histocompatibility complex, class II, DR beta 1	7.94	Up	Up
MMP7	matrix metallopeptidase 7 (matrilysin, uterine)	7.91	Up	Up
SLCO2A1	solute carrier organic anion transporter family, member 2A1	6.48	Up	Up
RBP1	retinol binding protein 1, cellular	6.04	Up	Up
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	4.70	Up	Up
BCL11A	B-cell CLL/lymphoma 11A (zinc finger protein)	3.66	Up	Up
LEMD1	LEM domain containing 1	3.40	Up	Up
AXIN2	axin 2	3.20	Up	Up
ST6GALNAC2	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 2	2.60	Up	Up
DACH1	dachshund homolog 1 (Drosophila)	2.45	Up	Up
SBK1	SH3-binding domain kinase 1	2.11	Up	Up
SOD2	superoxide dismutase 2, mitochondrial	-2.15	Down	Up
DUSP6	dual specificity phosphatase 6	-2.45	Down	Up
IL8	interleukin 8	-2.51	Down	Up
UPP1	uridine phosphorylase 1	-2.77	Down	Up
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1	-2.83	Down	Up
PIM1	pim-1 oncogene	-2.89	Down	Up
SERPINB9	serpin peptidase inhibitor, clade B (ovalbumin), member 9	-2.98	Down	Up
CPM	carboxypeptidase M	-3.00	Down	Up
FAM148A	C2 calcium-dependent domain containing 4A	-3.06	Down	Up
BMP6	bone morphogenetic protein 6	-3.24	Down	Up
SPTBN1	spectrin, beta, non-erythrocytic 1	-3.50	Down	Up
EDNRB	endothelin receptor type B	-3.53	Down	Up
GADD45A	growth arrest and DNA-damage-inducible, alpha	-3.61	Down	Up

UGCG	UDP-glucose ceramide glucosyltransferase	-4.38	Down	Up
CTSL2	cathepsin L2	-4.62	Down	Up
VCAN	versican	-4.86	Down	Up
PLAUR	plasminogen activator, urokinase receptor	-4.92	Down	Up
ITGA2	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	-6.28	Down	Up
VNN1	vanin 1	-9.47	Down	Up
ii) -H < +H equivalent to Post-M < Pre-M				
HSD17B2	hydroxysteroid (17-beta) dehydrogenase 2	-26.88	Down	Down
SLC3A1	solute carrier family 3 (cystine, dibasic and neutral amino acid transporters, activator of cystine, dibasic and neutral amino acid transport), member 1	-13.82	Down	Down
SLC30A2	solute carrier family 30 (zinc transporter), member 2	-8.56	Down	Down
COL1A2	collagen, type I, alpha 2	-7.56	Down	Down
IDH1	isocitrate dehydrogenase 1 (NADP+), soluble	-6.38	Down	Down
MGST1	microsomal glutathione S-transferase 1	-5.77	Down	Down
SLC39A14	solute carrier family 39 (zinc transporter), member 14	-5.76	Down	Down
HGD	homogentisate 1,2-dioxygenase	-5.20	Down	Down
TFPI2	tissue factor pathway inhibitor 2	-4.88	Down	Down
STEAP4	STEAP family member 4	-4.87	Down	Down
GNG11	guanine nucleotide binding protein (G protein), gamma 11	-4.82	Down	Down
SLC39A8	solute carrier family 39 (zinc transporter), member 8	-4.45	Down	Down
SEC14L1	SEC14-like 1 (S. cerevisiae)	-4.33	Down	Down
TAP2	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	-4.07	Down	Down
CKB	creatine kinase, brain	-4.05	Down	Down
ACSM3	acyl-CoA synthetase medium-chain family member 3	-3.86	Down	Down
KCNG1	potassium voltage-gated channel, subfamily G, member 1	-3.66	Down	Down
EAF2	ELL associated factor 2	-3.07	Down	Down
CYB5A	cytochrome b5 type A (microsomal)	-2.80	Down	Down
LRRC1	leucine rich repeat containing 1	-2.77	Down	Down
DLG5	discs, large homolog 5 (Drosophila)	-2.64	Down	Down
OGFOD1	2-oxoglutarate and iron-dependent oxygenase domain containing 1	-2.53	Down	Down
PELO	pelota homolog (Drosophila)	-2.49	Down	Down
SLC44A3	solute carrier family 44, member 3	-2.49	Down	Down

FGL1	fibrinogen-like 1	-2.48	Down	Down
AKAP13	A kinase (PRKA) anchor protein 13	-2.13	Down	Down

Differentially expressed genes between Pre-M and Post-M endometrial epithelial cells of the present study were found similar and in common with endometrial glands of explants cultured with (+H) and without (-H) hormones respectively. Fold change shown here were generated by Partek software analysing microarray data of this study. Comparative observation for each gene between this study and Gaide Chevronnay et. al. 2010 is indicated.

Chapter 4

N-cadherin isolates putative human endometrial epithelial stem/progenitor cells

Declaration

Monash University

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Responsible for refining the research question, performing the experiments, collecting and analysing and generating data for all figures except Figure 1, and manuscript writing	80 %

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Carl Sprung	Performed the initial microarray data analysis from raw data and helped revise the manuscript	10 %
Caroline Gargett	Conception and design of the study, helped revise the manuscript, read and approved the final manuscript	10 %

Candidate's Signature	[redacted]	**Date** 9 Dec 2011

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	The Ritchie Centre, Monash Institute of Medical Research Department of Obstetrics and Gynaecology, Monash University Monash Medical Centre, 246 Clayton Road, Clayton, Victoria, 3168, Australia

[Please note that the location(s) must be institutional in nature, and should be indicated here as a department, centre or institute, with specific campus identification where relevant.]

Signature 1	[redacted]	**Date** 9/12/11
Signature 2		9/12/11
Signature 3		

Abstract

Endometrial epithelial stem/progenitor cells postulated to reside in the basalis layer are responsible for the remarkable regeneration of the human endometrium each menstrual cycle. Retrospective analyses on human endometrium have identified rare clonogenic epithelial cells that exhibit stem cell properties: self-renewal, differentiation and high proliferative potential. A recent gene profiling study of pre- and post-menopausal endometrial epithelial cells revealed several potential candidate surface markers that may enrich for rare endometrial epithelial stem/progenitor cells. This study aims to determine if one of these markers, N-cadherin prospectively isolates the human endometrial epithelial stem/progenitor cell population using magnetic bead sorting. N-cadherin^{pos} and N-cadherin^{neg} endometrial epithelial cell populations were analysed for clonogenicity, proliferative potential, and their location within the endometrial epithelial compartment. N-cadherin^{pos} cells demonstrated higher clonogenicity, self-renewal and proliferative potential compared to N-cadherin^{neg} cells. N-cadherin^{pos} clones expressed cytokeratin and had high nuclear: cytoplasmic ratio, a feature of stem/progenitor cells. There was a gradient of N-cadherin expression on epithelial cells which was greatest in the basalis and diminished in the functionalis. This study demonstrates that N-cadherin is a putative marker that enriches for endometrial epithelial stem/progenitor cells reside in the basalis layer. This finding will enable investigation into the role of stem/progenitor cells in gynaecological disorders.

4.1 Introduction

The human endometrium, comprising two layers, functionalis and basalis, is a highly regenerative mucosa that grows 4-7 mm within 4-7 days during the proliferative phase of the menstrual cycle (Jabbour *et al.*, 2006; Gargett, 2007). Cyclical proliferation and

differentiation of the endometrium are regulated by fluctuating levels of circulating ovarian steroid hormones, estrogen and progesterone, respectively. It has been postulated that epithelial stem/progenitor (ESP) cells residing in the basalis layer are responsible for this monthly endometrial epithelial regeneration (Prianishnikov, 1978; Padykula *et al.*, 1989; Padykula, 1991; Gargett, 2007). A tritiated thymidine labelling study showed that the basalis had significantly slower cellular proliferation rates compared to the functionalis at all stages of the human menstrual cycle (Ferenczy *et al.*, 1979).

The first studies to report the existence of endometrial ESP cells used *in vitro* assays which identified rare clonogenic epithelial cells at all stages of the menstrual cycle, as well as in the thin, atrophic endometrium of post-menopausal women (Chan *et al.*, 2004; Schwab *et al.*, 2005). *In vitro* serial cloning study, the rare clonogenic cells initiating large clones demonstrated adult stem cell activity: self renewal, high proliferative potential with approximately 34 population doublings and differentiated into large cytokeratin-positive gland-like structures (Gargett *et al.*, 2009). Several studies have demonstrated a small side population (SP) cells as putative endometrial ESP cells that exhibited some adult stem cell properties (Kato *et al.*, 2007; Cervelló *et al.*, 2010; Masuda *et al.*, 2010). In particular, SP cells reconstituted endometrial tissue when transplanted *in vivo* (Cervelló *et al.*, 2010; Masuda *et al.*, 2010). Bone marrow-derived cells may also contribute to endometrial tissue regeneration (Taylor, 2004; Bratincsak *et al.*, 2007) and some descriptive morphological studies speculate that ESP cells may originate from endometrial stromal cells (Garry *et al.*). Given that endometrial ESP cells may originate from several sources and that their characterisation is retrospective, it is essential to identify candidate markers of endometrial ESP cell to determine their location and characterise their molecular and cellular properties.

A recent gene profiling study comparing freshly isolated, highly purified epithelial cells from pre-menopausal (Pre-M) and post-menopausal (Post-M) endometrial samples identified differential gene expression including several members of the Wnt signalling pathway (Nguyen *et al.*, 2011). Wnt signalling is important in stem cell fate determination during development (Logan & Nusse, 2004) and may regulates the basalis epithelial stem cell niche where the endometrial ESP cells likely reside. N-cadherin (*CDH2* gene, one of the Wnt-associated molecules identified in Chapter 3), is a calcium dependent cell adhesion molecule and member of the cadherin superfamily (Yagi & Takeichi, 2000; Derycke & Bracke, 2004). N-cadherin has an important role in the haematopoietic stem cell niche (Puch *et al.*, 2001; Zhang *et al.*, 2003) and has been used to isolate limbal epithelial stem/progenitor cells (Hayashi *et al.*, 2007; Higa *et al.*, 2009). Thus, we hypothesised that N-cadherin is a potential candidate marker that can be used to prospectively isolate the human endometrial ESP cell population.

The evidence generated in this study suggests that the N-cadherin marker enriches for an endometrial epithelial cell population with clonogenic activity and self-renewal capacity and locates these cells in the basalis layer. These findings indicate that N-cadherin may prospectively isolate endometrial epithelial stem/progenitor cells located in the basalis layer of the human endometrium.

4.2 Materials and Methods

4.2.1 Patient data

Human endometrial specimens (n = 44, 16 proliferative, 14 secretory and 14 post-menopausal) were obtained from women undergoing hysterectomy. Ethical approval was obtained from Southern Health Human Research and Ethics Committee B and Monash

University Human Research Ethics Committee with informed written consent given by each patient. A small portion of the tissue was embedded in OCT or formalin-fixed, paraffin embedded and assessed by an independent pathologist for histo-pathological evaluation and menstrual cycle dating according to Noyes's criteria (Noyes *et al.*, 1975) (Table 4.1). Pre-menopausal samples were taken from normal cycling women (30 – 52 years old) who had not taken steroid hormones within the 3 months prior to surgery. Post-menopausal samples were obtained from women (49 – 70 years old) who had not menstruated for at least 1 year prior to surgery nor had taken hormones (Nguyen *et al.*, 2011).

4.2.2 Endometrial epithelial cell isolation

The human endometrium was mechanically digested to single cell suspensions mechanically using enzyme cocktails as previously described (Nguyen *et al.*, 2011) with minor modifications. Briefly, the endometrium was scrapped off the myometrium, minced finely and digested in 10 ml culture medium (sodium bicarbonate buffered DMEM-F12, 10% fetal calf serum, 1% antibiotics and 1% glutamine (Invitrogen, Carlsbad, CA, USA), 50 mg/ml collagenase I (Worthington Biochemical, Freehold, NJ, USA) and 40 µg/ml DNase I (Worthington Biochemical) using a MACSmix rotator (Miltenyi Biotec, Bergisch Gladbach, Germany) for 90 min at 37ºC in a 5% CO_2 humidified incubator. The digestion was stopped and filtered through a 40 µm sieve. Glandular fragments containing epithelial cells remaining on the filter were backwashed and further digested for 20 min in 20 mg/ml collagenase II (Worthington Biochemical) and 40 µg/ml DNase I (Worthington Biochemical). Epithelial cell suspensions were then filtered through a 40 µm sieve and centrifuged for 5 min at 209 g and the pellets were resuspended in 5 ml medium. Cell counts were performed using a haemocytometer.

4.2.3 Epithelial cell selection and RNA extraction

Endometrial epithelial cell suspensions were further purified using anti-human EpCAM (BerEP4 clone) antibody-coated magnetic Dynabeads Epithelial Enrich kit (Invitrogen) according to the manufacturer's instruction and as described (Nguyen *et al.*, 2011). Briefly, the appropriate number of washed epithelial Dynabeads (4 beads/cell) were mixed with the backwashed cell suspension and incubated for 30 min at 4ºC with gentle mixing. Following incubation, the cell-bead suspension was placed on the magnet for 5 min to select beaded cells. The cell-bead suspension was washed twice and the beaded $EpCAM^{pos}$ epithelial cells were resuspended in PBS.

Total RNA was extracted from $EpCAM^{pos}$ epithelial cells using Ambion RNAqueous Micro Kit (Ambion, Applied Biosystems, Foster City, CA, USA), as previously described (Nguyen *et al.*, 2011). RNA preparations were DNase treated and purified using DNase Inactivation Reagent (Ambion). RNA purity and integrity was analysed by A260/A280 nm ratio using a NanoDrop ND1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware USA) and stored at -80ºC until use (Nguyen *et al.*, 2011).

TABLE 4.1 Details of patient samples used in this study

Sample number	Sample ID	Age	Menstrual Stage	Indication/Diagnosis	Assay
1	98-09	55	Post-menopausal	Fibroids	M
2	54-10	49	Post-menopausal	Unknown	M, Q
3	29-10	57	Post-menopausal	Prolapse	M
4	109-09	48	Proliferative	Menorrhagia	M
5	95-09	52	Proliferative	Prolapse	M, Q
6	96-09	49	Proliferative	Menorrhagia	M, Q
7	120-09	52	Proliferative	Fibroids	M, Q
8	122-09	45	Secretory	Menorrhagia	M, Q
9	88-09	30	Secretory	Severe endometriosis	M, Q
10	90-09	48	Secretory	Menorrhagia	M, Q
11	115-09	48	Secretory	Fibroids	M, Q
12	237-10	47	Proliferative	Menorrhagia	Q
13	246-10	36	Proliferative	Menorrhagia	Q
14	3-11	49	Proliferative	Fibroids	Q
15	5-11	40	Proliferative	Fibroids	Q
16	6-11	41	Secretory	Prolapse	Q
17	238-10	41	Secretory	Prolapse	Q
18	243-10	47	Secretory	Prolapse	Q
19	245-10	46	Secretory	Menorrhagia	Q
20	188-10	53	Post-menopausal	Prolapse	Q
21	240-10	54	Post-menopausal	Unknown	Q
22	7-11	64	Post-menopausal	Prolapse	Q
23	33-11	63	Post-menopausal	Prolapse	Q
24	35-11	53	Post-menopausal	Prolapse	Q
25	39-11	65	Post-menopausal	Prolapse	Q
26	44-11	66	Post-menopausal	Prolapse	Q
27	46-11	50	Post-menopausal	Prolapse	Q
28	48-11	54	Post-menopausal	Prolapse	Q
29	7-03	47	Proliferative	Menorrhagia	I
30	91-05	51	Proliferative	Menorrhagia	I
31	91-04	42	Secretory	Leiomyoma	I
32	161-04	38	Secretory	Adenomyosis	I
33	229-04	48	Secretory	Menorrhagia	I
34	95-06	43	Secretory	Adenomyosis	I
35	190-10	65	Post-menopausal	Prolapse	I
36	196-10	70	Post-menopausal	Prolapse	I
37	12-11	39	Secretory	Menorrhagia	F
38	32-11	46	Secretory	Menorrhagia	F
39	34-11	46	Proliferative	Menorrhagia	F
40	38-11	41	Proliferative	Leiomyoma	F, MS, C
41	45-11	48	Proliferative	Fibroids	F, MS, C
42	53-11	51	Proliferative	Adenomyosis, leiomyoma	F, MS, C
43	62-11	48	Proliferative	Leiomyoma	F, MS, C
44	64-11	45	Proliferative	Chronic endometritis	F, MS, C

M, microarray; Q, qRT-PCR; I, immunohistochemistry; F, flow cytometry sorting; MS, magnetic bead sorting for N-cadherin and P-cadherin; C, cloning assay. Samples 1-36 were the same patient samples used in Chapter 3.

4.2.4 Gene expression microarrays and analysis

Gene chip hybridisation, scanning and data acquisition was performed at the Australian Genome Research Facility (AGRF, Melbourne, Australia) as described (Nguyen *et al.*, 2011). Briefly, RNA samples (n = 11) were assessed for quality and integrity using Agilent Bioanalyser 2100 (Agilent, Santa Clara, CA, USA). Samples (500 ng) were labelled using the Ambion total prep RNA amplification kit (Ambion). A total of 1.5 µg of labelled cRNA was used to prepare a probe cocktail (0.05 µg/µl cRNA and GEX-HYB hybridisation buffer according to the manufacturer's protocol) and hybridised for 16 hr at 58ºC to a Sentrix Human-HT12 Beadchip (Illumina, San Diego, CA, USA). The chip was then washed, coupled with Cy3 and scanned using the Illumina BeadArray Reader (Illumina). BeadStudio scanner software (Illumina) was used to convert signal on the array to an appropriate file for analysis. The intensity of probe sets and batch normalisation were analysed using Partek Genome Suite 6.5 analysis package (Partek, St Louis, MO, USA). Differential gene expression was processed using robust multichip average (RMA) for background adjustment and analysis of variance (ANOVA) for statistical testing. Ingenuity Pathway Analysis software (IPA, Ingenuity Systems, Redwood City, CA, USA), DAVID (http://david.abcc.ncifcrf.gov) and GeneCards / Gene ALaCart (http://www.genecards.org) were used to obtain functional classifications and gene annotations (Section 3.2.5).

4.2.5 Transcription validation by quantitative RT-PCR

Primers (Table 4.2) were designed for candidate genes using public databases and "primer 3" software. RNA was reverse transcribed to cDNA using SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen) according to manufacturer's instructions with minor modifications. Quantitative RT-PCR was performed using SYBR Green PCR

Master Mix kit and 7900 HT Fast Real-Time PCR System (Applied Biosystems). Each reaction (100 ng cDNA, 1-50 µM of optimised primers and 1X Fast Power SYBR Green mix) was run in triplicate and the amplification conditions were 10 min at 95ºC, 15 sec at 95ºC and 1min at 60ºC. No-template and no primer-controls were included for each assay as quality assessment of cDNA specificity of the primers and PCR products were verified by agarose gel electrophoresis. Target gene expression was normalised to 18S rRNA. Relative gene expression was assessed using $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

TABLE 4. 2 List of primers used for qRT-PCR validation

Gene symbol	Forward primer (5' - 3')	Reverse primer (5' - 3')
CDH2	caacttgccagaaaactccagg	atgaaaccgggctatctgctc
CDH3	gctggggaaagtattcatggg	tcagggacagatattggagcaa
ITGB2	cagttattttccgccctcaa	acgcacctaacctcaccaac
ITGB4	gtcttctccaccgagtcagc	cgggtagtcctgtgtcctgt
EPHB4	gagctgtgtggcaatcaaga	atattggggtgctcgaactg
PROM2	gggacgagtgaagacagagc	tcttgggggacatcagagac
NOTCH4	gggtgagacgtgccagtttc	ctgggtgtcaatggagaggga
FZD2	aaggtgccatcctatctcagc	tagcagcccgacagaaaaatg
FZD9	gcggcaccaacacagagaa	agacatagcaaacgatgacgc

4.2.6 Flow cytometric analysis of human endometrial epithelial cells

Purified endometrial epithelial cells (1 x 10^5) were washed and resuspended in 100 µl cold flow washing buffer (phosphate buffered saline (PBS) and 2% fetal calf serum (FCS), Invitrogen, Australia). The cell suspension was blocked with 5% rat serum and incubated with primary antibodies; mouse anti-human N-cadherin (10 µg/ml, clone GC-4, Abcam, Cambridge, UK) or mouse anti-human P-cadherin (5 µg/ml, clone MM0508-9V11, Abcam) or the same concentration of isotype-matched control, mouse anti-human IgG_1 (Invitrogen) for 45 min at 4ºC. The cell suspension was washed with cold flow washing buffer, and centrifuged for 4 min at 209 g. Tubes were blocked with 5% rat serum and

labelled with secondary antibody, PE- conjugated rat anti-mouse IgG_1 (2 µg/ml, Invitrogen) for 30 min at 4ºC. Contaminated stromal, leukocytes and endothelial cells were removed by incubated with 5% mouse serum, followed by APC-conjugated mouse anti-human CD90 (10 µg/ml, Invitrogen), APC-conjugated anti-human CD31 (10 µg/ml, Invitrogen) and PeCy5.5-conjugated mouse anti-human CD45 (10 µg/ml, Invitrogen) antibodies for 30 min at 4ºC. The cell suspensions were washed and resuspended in cold flow wash buffer containing propidium iodide (PI, 10 µg/ml, Sigma Aldrich, St Louis, MO, USA) prior to flow cytometric analysis.

Data acquisition of fluorescently labelled cells was performed and analysed using MoFlo BTA analyser SUMMIT software, version 5.01 (Dako Cytomation, Fort Collins, CO, USA). Viable cells were selected based on forward scatter (FSC) and side scatter (SSC) dot plots of the cell suspension, which were then used to electronically gate out $CD90^+CD31^+CD45^+$ (stromal, endothelial and leukocytes) and PI^+ dead cells. The isotype-matched control stained cells were used to set electronic gates for each antibody and the percentage of positive N-cadherin or P-cadherin epithelial cells determined.

4.2.7 Magnetic bead cell sorting of human endometrial epithelial cells

Purified human endometrial epithelial cell suspensions (1 - 2 x 10^6) were washed with washing buffer (0.1% bovine serum albumin (BSA) in PBS, Invitrogen) according to the manufacturer's protocols and labelled with antibodies against mouse anti-human N-cadherin (10 µg/ml) or mouse anti-human P-cadherin (5 µg/ml) for 30 min at 4ºC. Following incubation, the cell suspension was washed and centrifuged for 8 min at 300 g. Pan anti-mouse IgG Dynal beads (25 µl/10^6 cells, Invitrogen) were used to select for N-cadherin or P-cadherin positive cells. The cell-bead suspension was incubated on a slow

rotating MACSmix rotator (Miltenyi Biotec) for 20 min at 4ºC, allowing the beads to attach to the antibody-labelled cells. Following incubation, the cell-bead suspension was placed on the magnetic stand for 5 min to select for bead-bound cells (N-cadherin or P-cadherin positive cells) and the supernatant (N-cadherin or P-cadherin negative cells) was collected in a separate tube. The cell-bead suspension was washed thrice and the bead-bound antibody-labelled epithelial cells were resuspended in washing buffer and counted using a haemocytometer.

4.2.8 Stem cell functional assay

4.2.8.1 In vitro colony-forming assay

Colony forming assays were performed as described (Chan *et al.*, 2004) with minor modifications. Briefly, the magnetic bead separated populations of endometrial epithelial cells (N-cadherin^{pos}, N-cadherin^{neg}, P-cadherin^{pos} and P-cadherin^{neg}) were seeded in duplicate at 3 clonal densities (50, 100, and 200 cells/cm^2), into fibronectin-coated 100 mm Petri dishes (Becton Dickinson, NJ, USA) and cultured in culture medium (Sodium Bicarbonate buffered DMEM-F12, 10% fetal calf serum, 1% primocin, 1% glutamine and 50 ng/ml epidermal growth factor, Invitrogen) for 15 days. N-cadherin^{pos} cells were also grown on coverslips for later use in immunohistochemistry. Culture medium was changed every 7 days and individual colonies were monitored daily to ensure they were derived from single cells.

Cloning plates were terminated at 15 days by fixing with 10% buffered formalin for 10 min at room temperature (RT) (Amber Scientific, WA, Australia). Cloning plates were then stained with filtered Harris haematoxylin for 4 min (Amber Scientific) and Scott's tap water for 30 sec to visualise the colonies. Colonies (> 50 cells) were counted using Bio-

Rad ChemiDoc XRS Molecular Imager and analysed using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). Cloning efficiency was calculated as the number of colonies divided by the total number of cells seeded on the plate and multiplied by 100.

4.2.8.2 In vitro self-renewal assay

Self-renewal ability of N-cadherin cells was assessed by serial cloning of individual colonies initiated by single epithelial cells (CFU) as previously (Gargett *et al.*, 2009) with minor modifications. Briefly, well-separated individual epithelial clones (at least 3-5 clones/plate) were selected using TrypleLE Express (Invitrogen) in cloning rings (Sigma-Aldrich) and re-seeded at clonal density (5-10 cells/cm^2) to generate secondary clones. After 13-17 days in culture, three secondary clones were selected and re-seeded to generate tertiary clones. This serial sub-cloning was repeated until cloning activity was exhausted.

4.2.9 Immunohistochemistry

Cryosections (5 µm) of fresh frozen full-thickness endometrium or N-cadherin^{pos} cells grown on coverslips were washed twice with PBS and fixed with 100% cold acetone (Amber Scientific) for 10 min at RT and air dried for 20 min. Cells were washed and blocked with 0.3% hydrogen peroxidise at RT for 10 min, followed by protein blocking solution (Dako, CA, USA) for a further 10 min. Sections and coverslips were incubated with primary antibodies mouse anti-human N-cadherin (4 µg/ml) or P-cadherin (5 µg/ml) or mouse anti-human pan-cytokeratin (1:100, 180 mg/l Dako) and isotype-matched control, mouse IgG_1 at the same concentration for 1 hr at 37ºC. Sections and cells were washed twice with PBS and incubated with anti-mouse Dako Envision System kit (Dako) for 1 hr at RT. Sections and cells were incubated with diaminobenzidine (DAB) chromogen for 5

min at RT. Sections and cells were counterstained with filtered Harris haematoxylin for 30 sec, washed and mounted using Ultramount medium (Dako).

4.2.9.1 Dual colour immunofluorescence

Fresh frozen full thickness pre- and post-menopausal hysterectomy tissues were cryosectioned (5 µm), fixed in 4% paraformaldehyde/PBS for 20 min at RT and permeabilised with 0.2% Triton X-100/PBS for 10 min. Sections were washed and incubated with protein blocking solution (Dako) for 10 min, followed by overnight incubation at 4ºC with primary antibodies mouse anti-human N-cadherin (4 µg/ml) concurrently with rabbit anti-human Ki67 (1:200, clone SP6, Thermo Scientific). Sections were washed and sequentially incubated with secondary antibodies Alexa Fluor488-congubated-donkey anti-rabbit IgG (1:200, Molecular Probes, Oregon, USA) to visualise Ki-67 staining, and biotinylated polyclonal goat anti-mouse IgG (1:500, Invitrogen) to detect N-cadherin together for 1 hr at RT. Sections were washed and incubated with streptavidin-Cy3 (1:500, Invitrogen) for 1 hr at RT to visualise N-cadherin. Negative controls were stained similarly but without primary antibody. Sections were washed and counterstained with DAPI (1:1000, Invitrogen) for 15 min and mounted in fluorescence mounting media (Dako). All images were captured and analysed using Leica DMR microscope and Leica IM50 Image manager v1.20 (Leica Microsystems, Wetzlar, Germany) and Image J software (NIH, Berthesda, MD, USA).

4.2.9.2 Enumeration of N-cadherin^{pos} cells

The number of N-cadherin^{pos} cells, Ki67^{pos} cells present in the functionalis and basalis layer of proliferative and secretory Pre-M and Post-M endometrium were assessed using similar method described previously (Chan *et al.*, ; Chan & Gargett, 2006; Kaitu'u-Lino *et*

al., 2010). Briefly, for each section examined, all epithelial nuclei in the functionalis and basalis of proliferative and secretory Pre-M and Post-M endometrium were counted. Only strong immunoreactive cells were counted as positive and the number of nuclei ranged from 76 to 300 per gland per section area, totalling to an average of 1100 nuclei per section counted. N-cadherin or Ki67 labelled cells were calculated as percentage of positive expressing cells to the total epithelial cell nuclei and results are presented as mean and standard error of the mean (SEM).

4.2.10 Statistical analysis

Flow cytometry, cell cloning and qRT-PCR data were analysed using GraphPad Prism software (version 5.01, CA, USA). Flow cytometric data are presented as mean ± SEM while cell cloning and qRT-PCR data are presented as median with minimum and maximum range. Gaussian distribution was assessed using D'Agostino and Pearson omnibus normality test. Mann-Whitney U test was used for comparison between two groups and Kruskal-Wallis was used to compare three groups when applicable. Differences of $p < 0.05$ (95% confidence interval) were considered to be statistically significant. Microarray data was analysed by robust multichip average (RMA) and analysis of variance (ANOVA) using Partek Genome Suite 6.5 analysis package (Partek, St Louis, MO, USA). The criteria for filtering the gene list was $p < 0.05$ and fold change > 2.

4.3 Results

At present, there is no known surface marker that prospectively isolates endometrial epithelial stem/progenitor cells, thus the identification of candidate markers is essential to define, characterise and study their origin and location. A gene profiling study of

endometrial epithelial cells from Pre-M and Post-M generated a short-list of potential markers including N-cadherin and P-cadherin. Stem cell activity of N-cadherin and P-cadherin were examined to determine whether either would enriched for rare endometrial epithelial stem/progenitor cells.

4.3.1 Candidate markers of human endometrial epithelial stem/progenitor cells

A transcriptome study of pure, homogenous epithelial cells isolated from Pre-M and Post-M endometrium (Chapter 3) identified a panel of 22 candidate cell adhesion molecules and plasma membrane receptors that may serve as candidate surface markers of endometrial ESP cells (Fig. 4.1). Eleven genes showed higher expression and eleven genes showed lowered expression in Post-M compared to Pre-M endometrial epithelial cells. Interestingly, four candidate genes (*CDH2*, *CDH3*, *FZD2* and *FZD9*) were identified as part of the *Wnt signalling pathway* (Chapter 3).

Nine candidate genes (8 up- and 1 down-regulated) were validated by qRT-PCR analysis of pure endometrial epithelial cells (Fig. 4.2). Validation showed statistically significant differences between Pre-M and Post-M endometrium which was concordant with the microarray data. *CDH2*, *EPHB4* and *FZD2* ($p < 0.001$), *ITGB2*, *ITGB4*, *CDH3*, *NOTCH4* and *PROM2* ($p < 0.05$) were higher, while *FZD9* ($p < 0.05$) was lower in Post-M compared to Pre-M endometrial epithelial cells.

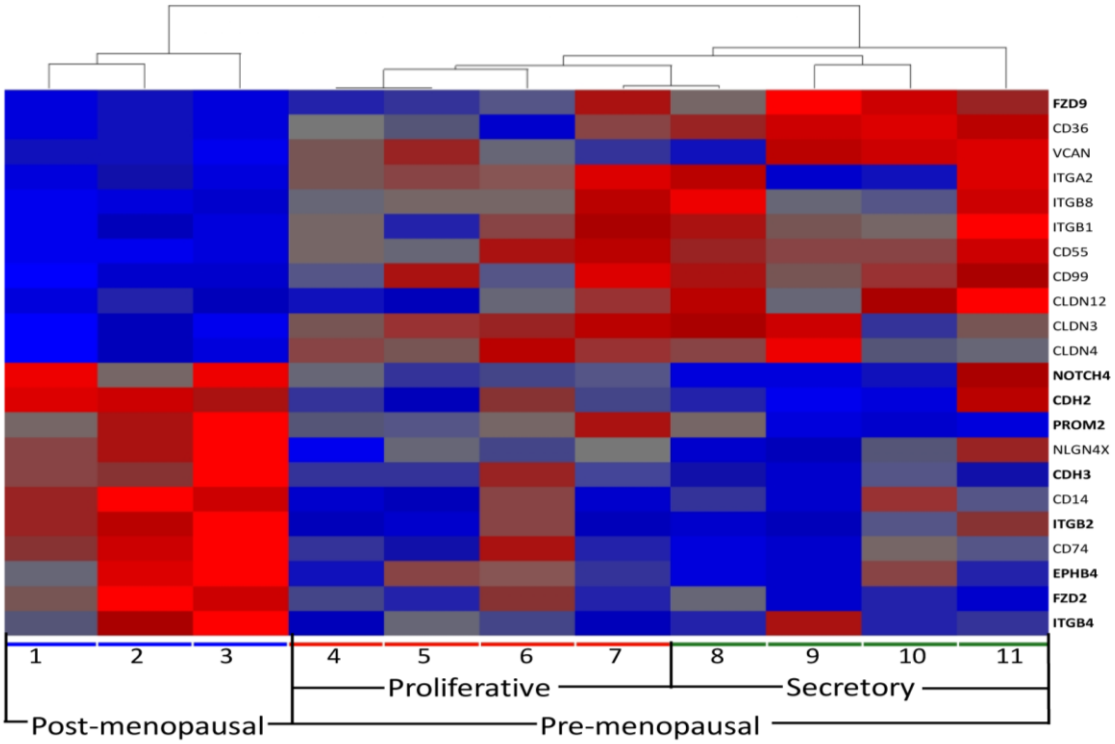


Figure. 4.1 Differential expression of candidate surface markers in Pre-M and Post-M endometrial epithelial cells. Hierarchical cluster analysis of 22 candidate cell adhesion molecules and plasma membrane receptors showing $p < 0.05$ (ANOVA) and a fold change of > 2 for comparison between Post-M (sample 1 – 3) and Pre-M proliferative (sample 4 – 7) and Pre-M secretory (sample 8 – 11) endometrial epithelial cells. Each column shows the relative gene expression of a single patient sample for the candidate surface markers which are labelled on the abscissa. Higher expression (red) and lower expression (blue) is indicated by colour and intensity. qRT-PCR validation of selected genes are identified by bold lettering. Dendrograms and heat map were generated by Partek Genomics Suite software.

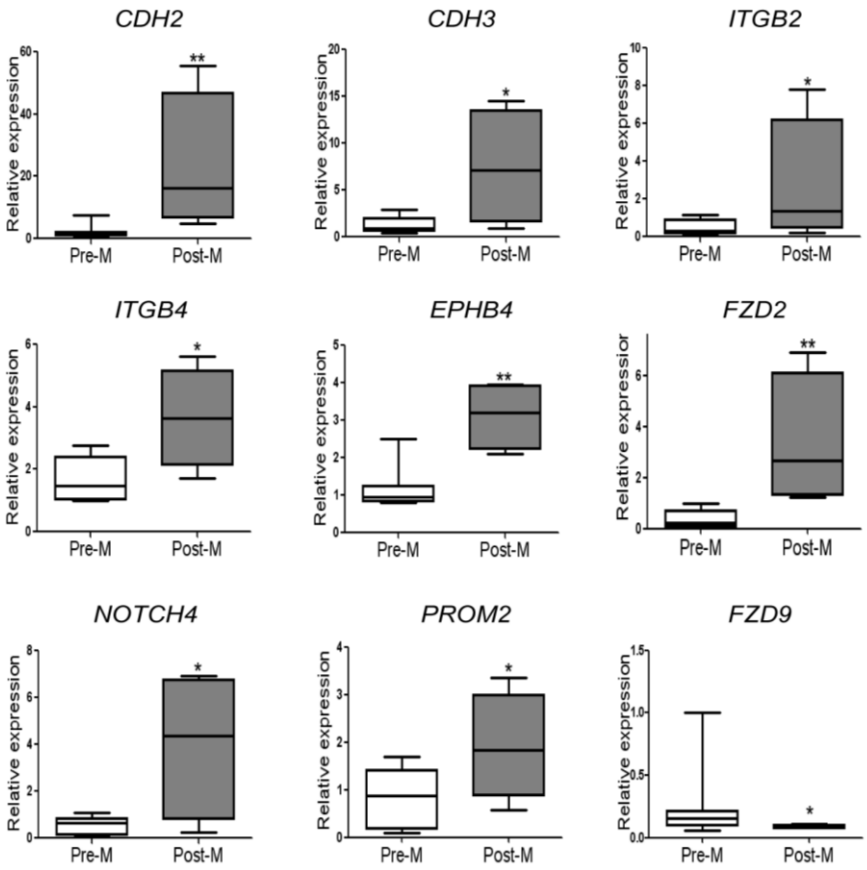


Figure. 4.2 Validation of 9 candidate cell adhesion molecules and plasma membrane receptors in Pre-M and Post-M endometrial epithelial cells by qRT-PCR. All 9 genes showed statistically significant differences in expression between Pre-M (n = 8, white bar) and Post-M (n = 4, grey bar). Relative expression was normalised to 18S. Data are presented as box and whisker plots showing medians and minimum and maximum range (95% confidence interval). **p < 0.001; * p < 0.05.

4.3.2 Phenotype and location of N-cadherin and P-cadherin protein in human endometrium

Given that N-cadherin (*CHD2* gene) and P-cadherin (*CDH3* gene) were found differentially expressed between Pre-M and Post-M endometrial epithelial cells and are associated with the Wnt signalling pathway (Chapter 3), they were chosen for further characterisation as potential candidates to prospectively isolate endometrial ESP cells.

The percentage of N-cadherin^{pos} endometrial epithelial cells was 19.2 ± 3.9% (n = 8) when analysed by flow cytometry sorting and 21.4 ± 4.4% (n = 5) when analysed by magnetic bead sorting (Fig. 4.3). Both approaches identified a similar percentage of N-cadherin^{pos} endometrial epithelial cells (p = 0.4).

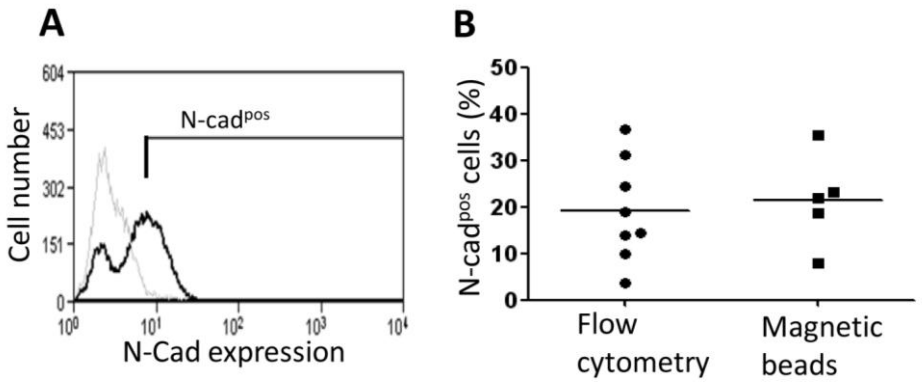


Figure 4.3 N-cadherin expression in endometrial epithelial cells. A) Flow cytometric histogram of N-cadherin expression. N-cadherin^{pos} cells are indicated by black line. The isotype-matched IgG control is shown in grey line. **B)** Percentage of N-cadherin^{pos} cells by flow cytometry sorting (n = 8) and magnetic bead sorting (n = 5). Bars are means. There was no statistically significant difference between the two sorting methods.

Immunofluorescence staining localised N-cadherin to the apical surface of most epithelial cells in the basalis (67.2%, n = 2) and not in the functionalis (0%, n =2) of proliferative phase (Fig. 4.4A and Fig. 4.5A, E). N-cadherin protein was highly concentrated in the deep basalis, adjacent to the myometrium comparing to all the other parts of the endometrium and the level of expression gradually decreased towards the functionalis (Fig. 4.4A). N-cadherin localised to epithelial cells in both the functionalis (70.4%, n = 2) and basalis (80.8%, n = 2) of secretory phase (Fig. 4.4B and Fig. 4.5I, M). N-cadherin stained prominently in Post-M endometrial epithelial cells (81.5%, n = 2) (Fig. 4.5R, S).

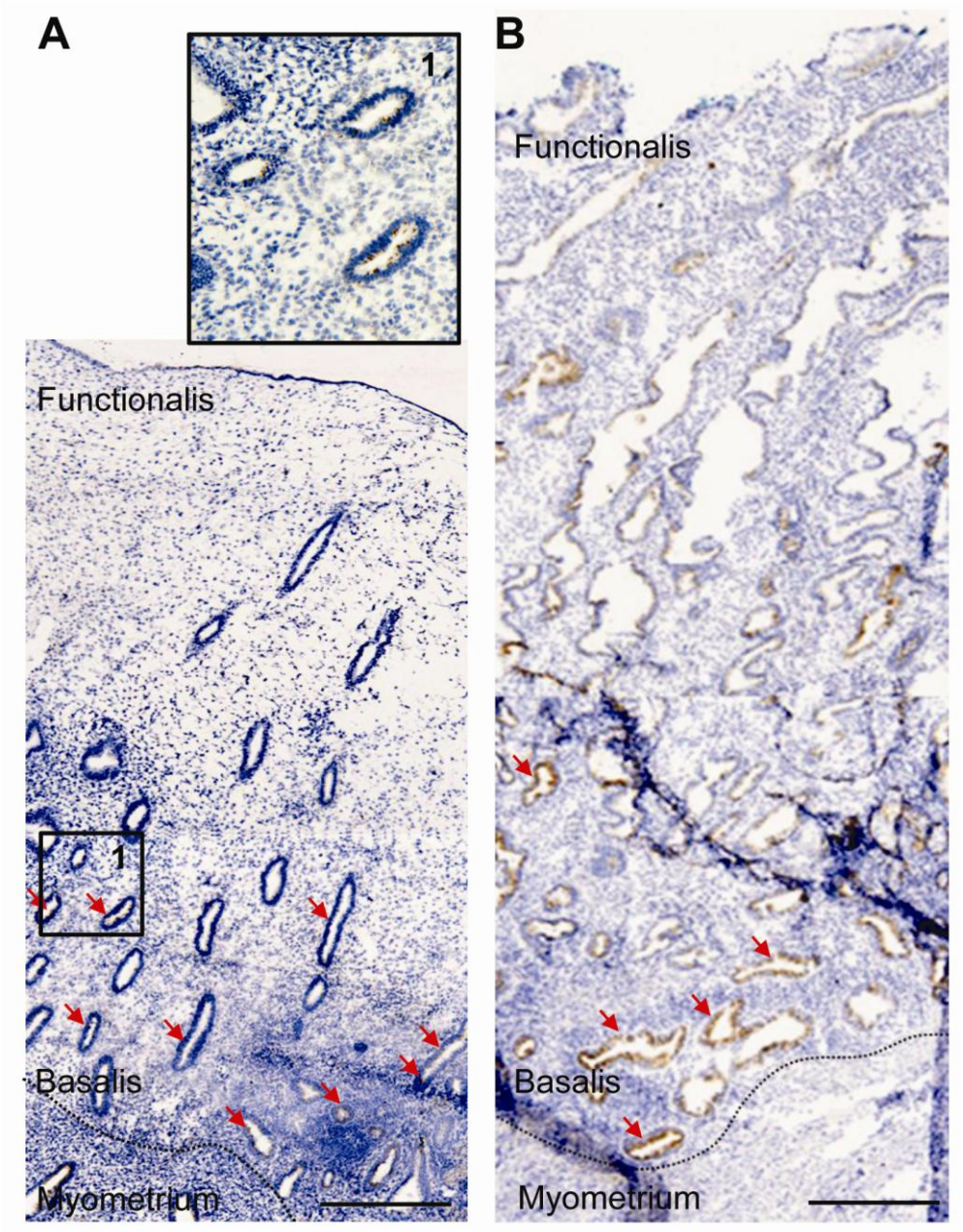
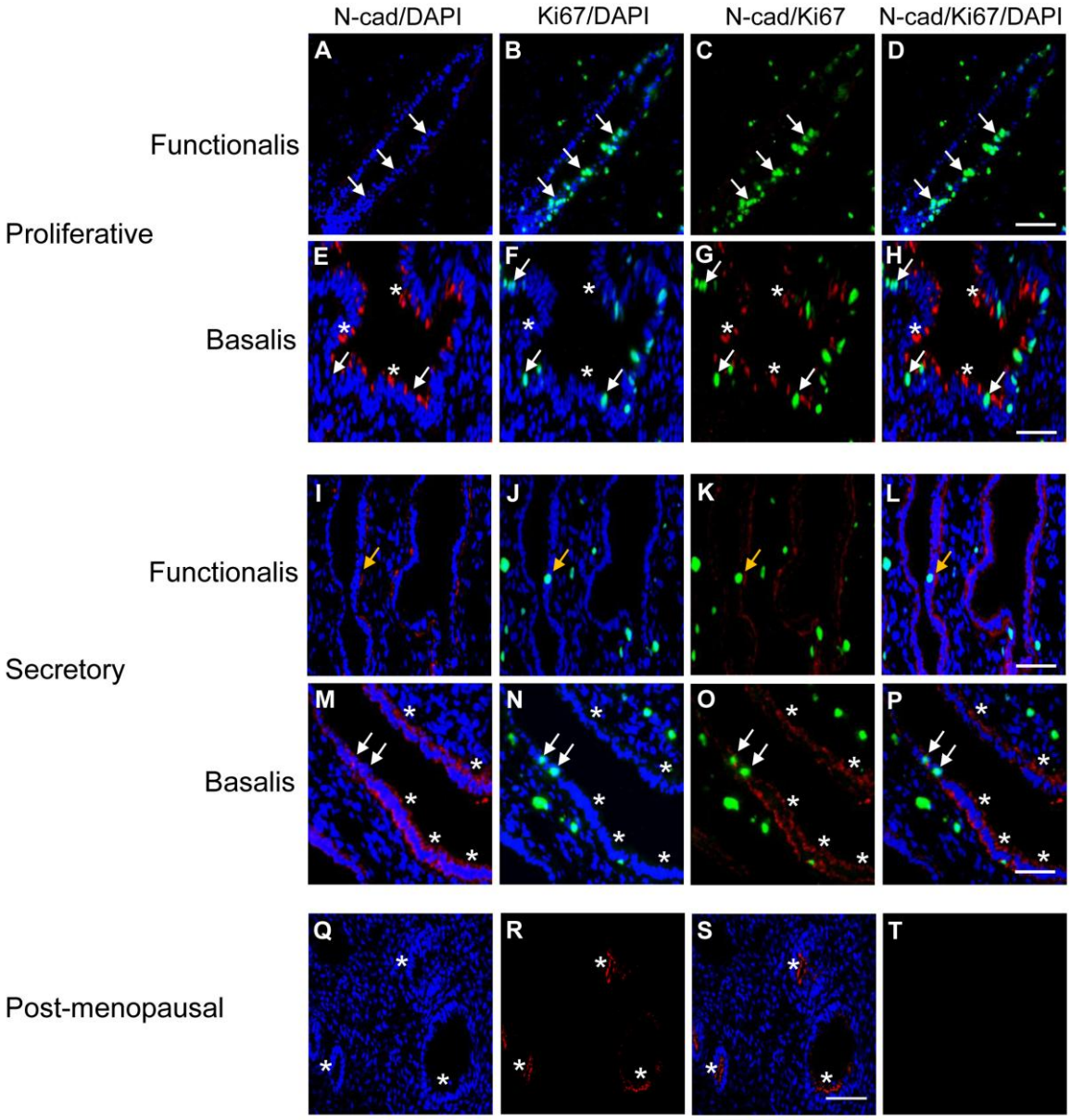


Figure. 4.4 Location of N-cadherin in full thickness endometrium of proliferative and secretory phase. N-cadherin is expressed in epithelial cells of the glands. Gland profiles showing greatest expression are indicated by red arrow. The expression is most prominent in the basalis of **A)** proliferative and **B)** secretory endometrium. The expression is low or absent in the functionalis of **A)** proliferative phase and less prominent in functionalis of **B)** secretory endometrium. **1.** Enlargement of N-cadherin positive expressing epithelial cells (brown staining). Scale bar = 200 µm**.**

Since N-cadherin distribution varied between basalis and functionalis layer, dual immunofluorescence for N-cadherin and Ki67 was employed to investigate whether N-cadherin^{pos} epithelial cells were quiescent as expected for stem/progenitor cells and whether their likely location was in the basalis. In the proliferative phase, the percentage of Ki67 positive epithelial cells was lower in the basalis (4.0 ± 0.8%, n = 4) compared to the functionalis (44.0 ± 3.1%, n = 4) (p = 0.0003) (Fig 4.5B, F). In the secretory phase, there was no difference in Ki67 positive epithelial cells in the basalis (1 ± 0.1%, n = 4) and functionalis (0.7 ± 0.04%, n = 4, p = 0.2) (Fig 4.5J, N). As expected, there was no co-localisation of N-cadherin (red) and Ki67 (green) in the basalis of both proliferative and secretory phase of the menstrual cycle (Fig 4.5H, P). However, N-cadherin appeared to co-localise with the occasional Ki67 positive epithelial cells in the functionalis of secretory phase (Fig. 4.5K). This data suggests that N-cadherin may be a marker of putative quiescent endometrial epithelial stem/progenitor cells.

This study also investigated the location of P-cadherin. P-cadherin localised to the baso-lateral surface of all epithelial cells in both functionalis and basalis of the proliferative and secretory Pre-M endometrium and Post-M endometrium (Supplemental Fig. 4.1A). P-cadherin was expressed by 2.5 ± 1.5% (n = 6) and 3.1 ± 1.3% (n = 4) of total endometrial epithelial cells when analysed by flow cytometric and magnetic bead sorting respectively (Supplemental Fig. 4.1B, C). The mean fluorescence intensity for P-cadherin was much lower in flow cytometry and magnetic bead sorting compared to immunohistochemistry, suggesting enzyme sensitivity of the epitope during the isolation procedure.

Figure. 4.5 Immunofluorescence of N-cadherin and Ki67 in full thickness proliferative and secretory Pre-M and Post-M endometrium. Co-localisation of N-cadherin (red) and DAPI (blue) *(first column)*, Ki67 (green) and DAPI *(second column),* Ki67 and N-cadherin *(third column)* and N-cadherin, Ki67 and DAPI *(fourth column)*. **A-D)** *White arrow*: only Ki67 but not N-cadherin (N-cadherin^{neg}Ki67^{pos}) staining is present in epithelial cells in the functionalis of the proliferative phase. **E-H)** *White star*:* N-cadherin^{pos}Ki67^{neg}, w*hite arrow:* N-cadherin^{neg}Ki67^{pos} epithelial cells in the basalis of proliferative phase. **I-L)** *Yellow arrow:* a few N-cadherin^{pos}Ki67^{pos} epithelial cells in the functionalis of secretory phase were observed. **M-P)** *White star*:* N-cadherin^{pos}Ki67^{neg} and w*hite arrow:* N-cadherin^{neg}Ki67^{pos} epithelial cells in the basalis of secretory phase. **Q-S)** W*hite star**: prominent N-cadherin^{pos}Ki67^{neg} staining in epithelial cells of post-menopausal endometrium. **T)** Control, secondary antibody only. Scale bar = 50 µm.


N-cad/DAPI
Ki67/DAPI
N-cad/Ki67
N-cad/Ki67/DAPI
Proliferative
Functionalis
Basalis
Secretory
Functionalis
Basalis
Post-menopausal
A
B
C
D
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4.3.3 Assessment of stem cell activity of N-cadherin and P-cadherin positive endometrial epithelial cells

Colony-forming assays were used to assess whether endometrial ESP cells were enriched in the magnetic bead sorted N-cadherin^{pos} or N-cadherin^{neg} cell fractions. Both N-cadherin^{pos} and N-cadherin^{neg} cells formed colonies when seeded at clonal density. Cloning efficiencies (CE) of N-cadherin^{pos} was 0.6% (range from 0.3% to 6.7%, n = 5) and N-cadherin^{neg} was 0.5% (range from 0.2% to 4.1%, n = 5) (Fig. 4.6A). While there was no significant difference (p = 0.09) between the CE of N-cadherin^{pos} and N-cadherin^{neg} clones (Fig. 4.6C, D), there was a trend toward higher CE for the N-cadherin^{pos} cells. Additionally, N-cadherin^{pos} colonies also stained positive for cytokeratin, a epithelial-specific marker (Fig. 4.6E) and the cells were small and compact with a high nuclear: cytoplamic ratio, a property of stem cells (Chan *et al.*, 2004).

To determine the self-renewal activity, a key property of stem cells, of N-cadherin^{pos} and N-cadherin^{neg} cells, serial cloning was undertaken. N-cadherin^{pos} cells serially cloned ≥ three times while N-cadherin^{neg} cells failed to re-clone (Fig. 4.6B). There was a trend for increasing cloning-forming unit (CFU) in N-cadherin^{pos} secondary and tertiary clones. This is in line with large clones of earlier study (Gargett *et al.*, 2009). This data indicates that N-cadherin^{pos} but not N-cadherin^{neg} CFU undergoes self-renewal *in vitro* and suggestive of stem/progenitor cells population.

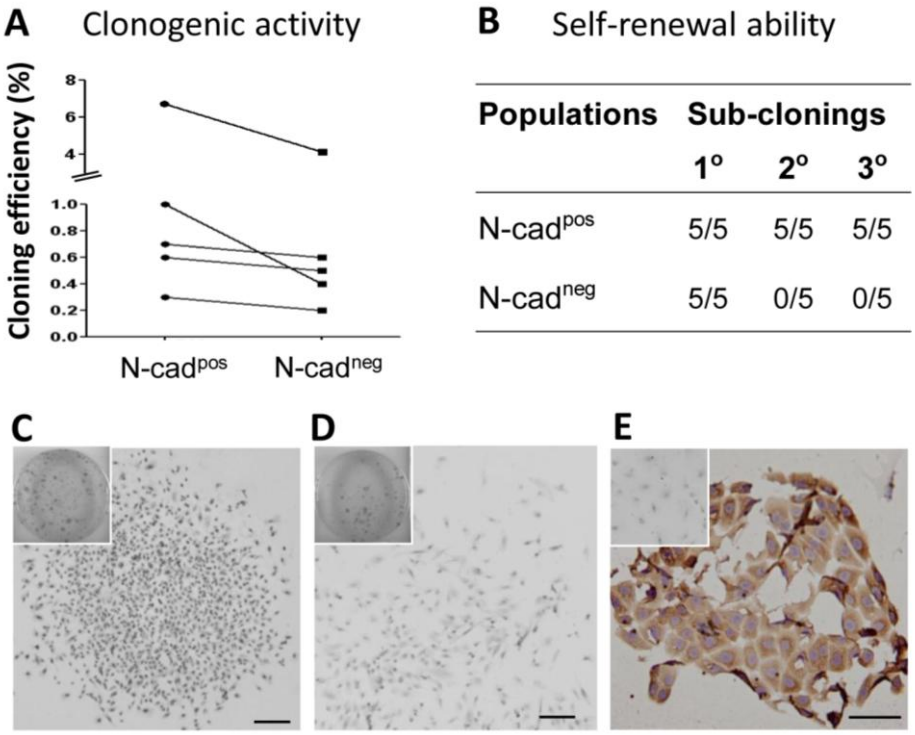


Figure 4.6 Functional studies of N-cadherin^{pos} and N-cadherin^{neg} human endometrial epithelial cells. **A)** Colony-forming ability of magnetic bead sorted N-cadherin^{pos} and N-cadherin^{neg} cell populations (n = 5). **B)** Serial cloning analysis for measuring self-renewal of N-cadherin^{pos} and N-cadherin^{neg} cells. Initial seeding density was 50 cells/cm^2 and individual primary clones were re-cloned at 10 cells/cm^2. Data are presented as the number of samples producing clones per number of samples examined for each re-cloning step. A minimum of 5 clones/patient samples was examined. Morphological features of typical **C)** N-cadherin^{pos} and **D)** N-cadherin^{neg} colonies-forming cells, insets: cloning plates. **E)** Clones initiated by N-cadherin^{pos} cells were positive for epithelial marker, cytokeratin. Staining showed small compact cells with high nuclear:cytoplasmic ratio, a feature of stem/progenitor cells, insets: N-cadherin^{pos} cells were unstained in isotype-matched negative control, IgG_1. Scale bar = 200 µm.

Both P-cadherin^{pos} and P-cadherin^{neg} had the ability to initiate and form colonies. Clonal efficiency of P-cadherin^{pos} was 0.4% (range from 0.1% to 7.9%) and P-cadherin^{neg} was 0.9% (range from 0.5% to 5.6%) (Supplemental Fig. 4.1D). However, neither P-cadherin^{pos} nor P-cadherin^{neg} cells were able to undergo self-renewal (data not shown). This data indicates that P-cadherin is not a suitable candidate marker of human endometrial ESP cells.

4.4 Discussion

The main finding of this study was the identification of N-cadherin as a potential marker of endometrial epithelial stem/progenitor cells. N-cadherin^{pos} cells, comprising 20% of endometrial epithelial cells, were enriched for clonogenic, self-renewing epithelial cells compared to N-cadherin^{neg} cells, although significance was not reached. However, the mean CE of N-cadherin^{pos} cells (1.8 %) was significantly higher ($p < 0.004$) than an earlier study of an unfractionated human endometrial epithelial cells (CE = 0. 22%) (Chan *et al.*, 2004). Thus, N-cadherin^{pos} CFU were enriched 8-fold compared to the unfractionated freshly isolated clonogenic endometrial epithelial cells (Chan *et al.*, 2004), indicating enrichment for clonogenic epithelial cells. The morphology of the N-cadherin^{pos} clones was similar to previously described for large epithelial clones (Chan *et al.*, 2004; Gargett *et al.*, 2009), cytokeratin expressing cells showing high nuclear: cytoplasmic ratio, another feature of cultured adult stem cells. N-cadherin was localised to majority of epithelial cells in the basalis but not in the functionalis of the proliferative phase, and in post-menopausal of the human endometrium. N-cadherin expression in the functionalis and basalis of secretory phase was similar. Another main finding of this study was that N-cadherin rarely co-localised with Ki67 proliferating cells regardless of menstrual cycle stage. N-cadherin identified a small subset of endometrial epithelial cells exhibiting clonogenicity, self-renewal, quiescence, and they located to the basalis layer, suggesting they may be endometrial epithelial stem/progenitor cells.

It is likely that human endometrial ESP cells predominantly reside in the basalis layer of human endometrium (Gargett, 2007). Our microarray, qRT-PCR and immunohistochemistry data showed more abundant expression of N-cadherin mRNA and protein in the basalis-like Post-M endometrium than in full thickness proliferative and

secretory Pre-M endometrial epithelial cells. Immunofluorescence staining revealed differential expression and localisation of N-cadherin in proliferative and secretory Pre-M and Post-M endometrium. N-cadherin staining intensity was more prominent in epithelial cells of the basalis compared to the functionalis layer of the endometrium. N-cadherin and Ki67 cells did not co-localised in the basalis layer. Only a few rare N-cadherin^{pos}Ki67^{pos} epithelial cells localised in the functionalis of secretory phase. The N-cadherin^{pos}Ki67^{pos} cells may be the transit-amplifying cells undergoing rapid proliferation to differentiate into the mature epithelial cells. The N-cadherin^{pos}Ki67^{neg} cells located in the basalis are more likely epithelial stem/progenitor cells. However, this data is still preliminary and required further investigation. The evidence indicates that epithelial cell proliferation is tightly downregulated with progressive differentiation. It is also possible that N-cadherin is involved in the development of progenitor cells as they commit to differentiation (Puch *et al.*, 2001).

Currently, there is no known marker that identifies endometrial epithelial stem/progenitor cells and therefore knowledge of their origin and location is very limited. It has long been suggested that epithelial regeneration arise from stem/progenitor cells in epithelial glands located in the basalis layer (Padykula *et al.*, 1989; Padykula, 1991; Gargett, 2007). However, this theory was challenged by an alternative concept that endometrial epithelial cell outgrowth is a consequence of endometrial stromal cell differentiation (Baggish *et al.*, 1967; Garry *et al.*, 2009, 2010). Thus, it is imperative that a marker of endometrial epithelial stem/progenitor cells is identified. This will confirm their origin and their role in epithelial regeneration. Evidence shown in this study demonstrated that N-cadherin could be used to partially purify and enrich for endometrial epithelial stem/progenitor cells.

Little is known about the distribution of N-cadherin in cycling and non-cycling endometrium. One study reported that N-cadherin is localised to epithelial glands throughout the menstrual cycle and some stromal cells of the secretory phase (Poncelet *et al.*, 2002). However the staining level was reported in arbitrary scoring units and no immunological staining was shown, making it difficult to determine the exact location of N-cadherin. Nevertheless, our present study confirms that epithelial cells express N-cadherin protein. Another study on infertile women reported the weak expression of N-cadherin on the apical-lateral plasma membrane of epithelial glands (Poncelet *et al.*, 2010). Given that N-cadherin is generally restricted to the basalis epithelial cells, its low detection rate may be due to the examination of endometrial biopsy. Nevertheless, the apical-lateral location of N-cadherin expression is in line with our current finding. The high N-cadherin protein level in the proliferative phase reported in an earlier study (Tsuchiya *et al.*, 2006) could likely be due to variation in N-cadherin transcript level. Alternatively, it is possible that individual patient sample profile and different techniques may contribute to different levels of N-cadherin expression in the endometrium reported here and that of previous reports.

N-cadherin previously identified haematopoietic stem cell niche (Puch *et al.*, 2001; Zhang *et al.*, 2003) and corneal limbal epithelial stem/progenitor cells (Hayashi *et al.*, 2007; Higa *et al.*, 2009). In the haematopoietic system, N-cadherin was found essential for mediating haematopoietic stem cell (HSC) association with osteoblasts, and which is a key component of the niche to support HSC (Zhang *et al.*, 2003). In the corneal system (Hayashi *et al.*, 2007; Higa *et al.*, 2009), N-cadherin^{pos} cells was found in the basal layer of limbal epithelium and co-localised with the hair follicle stem cell marker, K15. N-cadherin^{pos} cells showed higher mRNA levels of stem-cell related markers for ΔNp63,

K15, Bmi-1 and ABCG2. In addition, a major fraction of N-cadherin^{pos} cells was present in a slow cycling keratinocyte stem cell population ($\alpha6^{bri}$/CD71^{dim}) with higher clonal efficiency than N-cadherin^{neg} cells (Hayashi *et al.*, 2007). Taken together this evidence suggests N-cadherin may be a marker to partially purify and identify endometrial epithelial stem/progenitor cells.

The Wnt / β-catenin signalling pathway, particularly nuclear accumulation of β-catenin is involved in endometrial carcinoma progression (Fukuchi *et al.*, 1998; Moreno-Bueno *et al.*, 2002; Wang *et al.*, 2010). N-cadherin is a family member of cadherin-catenin complex that plays a pivotal role in cell-cell adhesion, cell motility and tumour progression including endometrial cancer (Hajra & Fearon, 2002; Singh *et al.*, 2011; Derycke & Bracke, 2004). A recent tissue array study found N-cadherin localised to endometrial cancer samples, in particular to non-endometrioid (type II) rather than endometrioid (type I) cancer (Singh *et al.*, 2011). In the present study, we report high levels of N-cadherin in post-menopausal endometrial epithelium. Our data and that of others suggest that N-cadherin may be an important adhesion molecule regulating β-catenin. Alterations in its signal transduction may leads to development of cancer (Husmark *et al.*, 1999). However, however, this concept merits further investigation in endometrial cancer.

While P-cadherin showed differential gene expression between proliferative and secretory Pre-M and Post-M endometrial epithelial cells, it did not appear to enrich for endometrial ESP cells. P-cadherin imunostained the baso-lateral surface of the whole gland profiles in both the functionalis and basalis of proliferative and secretory of Pre-M and Post-M endometrium, consistent with previous findings (van der Linden *et al.*, 1994; van der Linden *et al.*, 1995). P-cadherin^{pos} cells were not enriched for clonogenic epithelial cells not did they exhibit stem cell properties of self-renewal. The utitility of P-cadherin as a

potential endometrial ESP marker is further diminished because the epitope is enzyme sensitive, limiting its usefulness in cell separating procedures. Nevertheless, P-cadherin is essential for the maintenance of endometrial epithelial architecture and play a role in maintaining endometrial proliferation (van der Linden *et al.*, 1994).

Cell adhesion molecules are important in regulating stem cell behaviour and maintaining the stem cell within its niche (Jones & Wagers, 2008). Such markers have been used to isolate stem cell populations in other systems (Li *et al.*, 1998; Stingl *et al.*, 2001; Stingl, 2005) including endometrial mesenchymal stem-like cells (Schwab & Gargett, 2007; Schwab, 2008). In addition, large number of proteins present on the cell surface also assists technically when isolating rare cell populations. This was evident by the identification of a shortlist of cell adhesion molecules in our gene profiling study comparing full-thickness proliferative and secretory Pre-M and basalis-like Post-M endometrial epithelial cells (Chapter 3). In the endometrium, adhesion molecules are essential for cell-cell and cell-extracellular matrix interactions in preparation for embryo implantations (Singh & Aplin, 2009). They are also involve in re-epithelisation after menstruation as well as maintaining epithelial polarisation (Horne *et al.*, 2002). The cell adhesion molecules identified in this study (Fig. 4.1), particularly integrin molecules were higher in secretory phase, is consistent with previous studies (Lessey *et al.*, 1992; Tabibzadeh, 1992; van der Linden *et al.*, 1995). This present data highlights the importance of integrin molecules and other cell adhesion molecules in uterine receptivity.

Magnetic bead sorting was used in favour of flow cytometry sorting to purify and select a homogenous N-cadherin^{pos} cell population and examined stem cell activity. Although both magnetic bead and flow cytometry sorting yielded similar numbers of N-cadherin^{pos} endometrial epithelial cells, magnetic bead sorting was simpler and provided better viable

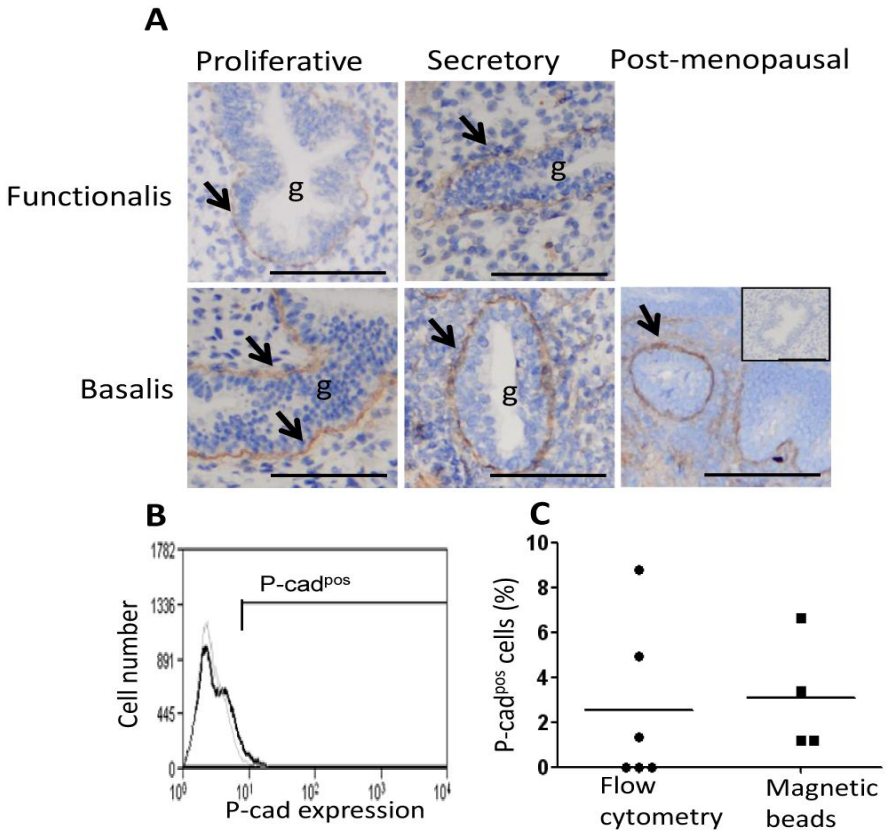
cell yields with shorter isolation time. Flow cytometry is a powerful method for analysing and sorting multiple sub-populations based on surface marker expression. It however requires large number of cells and access to highly sophisticated machine, software and trained personnel for operation. Furthermore, long cell isolation procedures contributed to cell damage and reduced cell viability (Geens *et al.*, 2007; Masuda *et al.*, 2011). Together with evidence of other studies, we suggest that magnetic sorting is the preferred method for isolating rare epithelial cell populations. Many protocols for other stem cells utilise several markers, the limitation of magnetic bead sorting is that multiple markers can only be used for negative selection.

One limitation of this study was the availability of sufficient human endometrial tissue samples, especially post-menopausal endometrium. The size limitation of post-menopausal endometrial samples, its thin and atrophic status limited the number of assays that could be done per sample. Clonogenic cells exist in Post-M endometrium (Schwab *et al.*, 2005), therefore clonogenicity of N-cadherin^{pos} Post-M epithelial cells should be investigated. To confirm the robustness of N-cadherin as a putative marker of endometrial ESP cells, *in vivo* endometrial epithelial tissue reconstitution should be examined. N-cadherin holds promise for the partial purification of endometrial ESP cells, however another marker may be needed because it is expected that the number of ESP cells should be lower than 20% of total endometrial epithelial cells.

There are currently no definitive markers for endometrial epithelial stem/progenitor cells. This study has prospectively isolated a small population of endometrial epithelial cells with stem cell functional properties for the first time. These rare cells are enriched in the N-cadherin^{pos} population and are found exclusively in the deep basalis layer, adjacent to the myometrial junction. Their distribution gradually decreased towards the functionalis and is

absent in the luminal epithelium. The present finding is significant in the field of endometrial stem cell biology. It provides an opportunity to extent further research into molecular and cellular characterisation of endometrial epithelial stem/progenitor cells. Additionally, the role of epithelial stem/progenitor cells in gynaecological disorders such as endometrial cancer and endometriosis can now be examined and elucidated.

4.5 Supplementary Information



D Clonogenic activity

Populations	Sample number		
	1	2	3
P-cad^{pos}	0.1	0.4	7.9
P-cad^{neg}	0.9	0.5	5.6

Supplemental Figure 4.1 Location of P-cadherin in full thickness Pre-M and Post-M endometrium. **A)** P-cadherin localised to the baso-lateral of endometrial glands in functionalis and basalis of proliferative (left panel) and secretory (middle panel) and Post-M (right panel). Scale bar = 100 µm, g: glands. Insets: isotype-matched control. **B)** Flow cytometric histogram of P-cadherin expression indicated by black line. Grey line: isotype-matched IgG control. **C)** Percentage of P-cadherin expression by flow cytometry sorting (n = 6) and magnetic bead sorting (n= 4). Bars are means. **D)** Colony-forming ability of magnetic bead sorted P-cadherin^{pos} and P-cadherin^{neg} cells. Results are from 3 individual samples.

Chapter 5

Candidate markers of human endometrial cancer stem cells

5.1 Introduction

Endometrial cancer (EnCa) is the seventh most common cancer worldwide and the most common gynaecological cancer (Di Cristofano & Ellenson, 2007) Of the two types of EnCa, Type I EnCa is more common and accounts for 80% of EnCa cases. It is classified as a low grade endometrioid carcinoma driven by unopposed estrogen stimulation and tends to arise from hyperplastic endometrium of peri-menopausal women (Bokhman, 1983; Di Cristofano & Ellenson, 2007). In contrast, type II EnCa arises from in atrophic endometrium of post-menopausal women in the absence of estrogen stimulation (Bokhman, 1983; Di Cristofano & Ellenson, 2007). Gene alterations also play a role in endometrial cancer. Mutations in PTEN, Kras, microsatellite instability or the Wnt / β-catenin pathway are common in type I endometrial cancer while mutations in p53 and Her2 is often seen in type II endometrial cancer (Latta & Chapman, 2002; Moreno-Bueno *et al.*, 2002; Di Cristofano & Ellenson, 2007).

Cancer stem cells (CSC) are defined as a small subset of tumour cells with similar properties to those of normal adult stem cells including the capacity to self-renew, high proliferative potential and differentiation (Reya *et al.*, 2001; Visvader & Lindeman, 2008), giving rise to the more differentiated cancer cells within the bulk of the tumour (Jordan *et al.*, 2006). It is thought that mutations occuring in the normal epithelial stem cells or their progeny contribute to the formation of CSC, particularly those which confer self-renewal. CSC are thought to be responsible for tumour initiation, maintenance and spreading, metastasis and recurrance (Pardal *et al.*, 2003). CSC were first studied in the leukaemias and subsequently have been identified in many solid cancers including breast, prostate, pancreas and brain. In these studies, different combinations of surface markers including EpCAM, CD24, CD44, CD49f (Al-Hajj *et al.*, 2003; Singh *et al.*, 2003; Li *et al.*, 2007)

(Table 1.1, Chapter 1) and flow cytometry sorting (FACS) were used to sort primary cancer cells into sub-populations for examining their relative ability to initiate tumour formation when transplanted into immuno-compromised mice.

Given that cells within the tissue express different surface markers, and that the presence or absence of these markers can isolate cancer stem cells in other organs (Table 1), it is possible that some of these markers could be used to isolate endometrial cancer stem cells. EpCAM is an epithelial cell adhesion molecule expressed in most carcinomas (Litvinov *et al.*, 1994; Baeuerle & Gires, 2007) and by virtually all endometrial epithelial cells was used to select epithelial cells from the dissociated endometrial cancer tissue. CD24 marker is a glycoprotein, adhesion molecule widely used to isolate different populations of CSC in hormone-regulated organs including the breast and ovary (Al-Hajj *et al.*, 2003; Gao *et al.*, 2010). CD44 is another adhesion molecule and a receptor for hyaluronan as well as collagens and osteopontin that is highly expressed in endometrial cancer (Fujita *et al.*, 1994; Afify *et al.*, 2005) and has been used widely to enrich for CSC in the breast, pancreas, prostate and colon (Al-Hajj *et al.*, 2003; Patrawala *et al.*, 2006; Li *et al.*, 2007; Vermeulen *et al.*, 2008). CD29 and CD49f are members of the integrin family that is important for stem cell environment (Watt, 2002). CD29 (also known as β1 integrin) when combined with CD24 *(a GPI-anchored glycoprotein involved in cell adhesion)* has been used to characterised mouse mammary epithelial stem cells (Shackleton *et al.*, 2006). CD49f (also known as α6 integrin) was used to isolate stem cells of the prostate and oesophagus (Croagh *et al.*, 2007; Lawson *et al.*, 2007).

Evidence for endometrial cancer stem cells has recently been reported (Friel *et al.*, 2008; Hubbard *et al.*, 2009). A small population of isolated EnCa cells initiated tumour formation *in vivo* and exhibited stem cell properties including colony-forming ability and self-

renewal. However, these studies were retrospective and the origin and existence of endometrial CSC remain unknown.

At the commencement of my candidature, no markers for endometrial cancer stem cells had been identified, therefore, this study aimed to examine several known stem cell surface markers from other tumours in primary endometrial cancer and endometrial cancer cell lines and to determine their potential to enrich for the human endometrial cancer stem cell population.

5.2 Materials and Methods

5.2.1 Human endometrial cancer tissue collection

Primary endometrial cancer (EnCa) samples were collected from 30 patients (range from 36 to 89 year old) undergoing hysterectomy (Table 5.1). Samples were collected by the Victoria Cancer Biobank with informed written consent obtained from each patient. Ethical approval was obtained from Southern Health and Monash University Human Research Committees.

Endometrial cancer cell lines; Type I: ECC1 was kindly donated by Prince Henry's Institute of Medical Research while Type I: Ishikawa line and Type II: HEC-1A line were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

5.2.2 Preparation of primary human endometrial cancer single cell suspensions

EnCa samples were processed within 6-8 hours of procurement and manually dissociated by finely mincing and digesting with 0.8 mg/ml collagenase II and 40 µg/ml DNase I

(Worthington Biochemical, Freehold, NJ, USA) in culture medium (Sodium bicarbonate buffered DMEM-F12, 10% fetal calf serum, 1% glutamine and 1% antibiotics, Invitrogen, Carlsbad, CA, USA) for 90 min at 37ºC with slow agitation. The digestion was stopped with 10 ml bench medium (DMEM-HEPES, 5% new born calf serum, 1% antibiotics, Invitrogen) and filtered through a 40 µm strainer. Remaining cell clumps were further digested with 0.8 mg/ml collagenase II and 40 µg/ml DNase I (Worthington) for 15 min with trituration at 5 min intervals, followed by filtering through a 40 µm strainer. Single cell suspensions from both fractions were combined, washed and pelleted using bench medium. A cell count was performed using a haemocytometer.

5.2.3 Flow cytometry sorting of purified endometrial cancer cells

Freshly isolated primary EnCa, ECC1, Ishikawa and HEC-1A cells ($1x10^5$ - $1x10^7$ cell/ml) were blocked with appropriate blocking serum, labelled with primary antibodies or control isotype-matched IgG control (Table 5.2) and incubated for 45 min at 4ºC. The cell-antibody suspension was washed with cold flow washing buffer (2% FCS/PBS) and incubated with the appropriate secondary antibodies for 30 min at 4ºC (Table 5.2). All tubes were incubated with anti- CD10-PE or CD90-APC, anti-CD45-PeCy5.5 and anti-CD31-PE to eliminate stromal cells, leukocytes and endothelial cells respectively. Cells were resuspended with 200 µl of flow buffer containing 7AAD (1 µg/ml, Invitrogen) and analysed or sorted using MoFlo-XDP flow cytometry sorter (Dako Cytomation, Inc., Fort Collins, CO, USA) and SUMMIT software (V5.01, Dako).

TABLE 5.1 Patient details used in this study

Sample number	Sample ID	Age	Type	Grade	Classification
1	08 SH 253	89	I	3	EC
2	08 SH 341	60	I	3	EC
3	08 SH 439	58	I	1	EC
4	08 SH 526	43	I	2	EC
5	08 SH 552	61	I	1	EC
6	08 SH 575	78	I	1	EC
7	08 SH 667	55	I	1	EC
8	08 SH 775	61	I	1	EC
9	08 SH 830	72	I	2	EC
10	08 SH 837	71	I	2	EC
11	08 SH 947	63	I	3	EC
12	08 SH 1021	50	I	2	EC
13	09 SH 655	67	I	2	EC
14	09 SH 691	58	I	2	EC
15	09 SH 735	64	I	2	EC
16	09 SH 785	69	I	2	EC
17	10 AH 357	77	I	1	EC
18	10 SH 422	36	I	1	EC
19	10 AH 534	68	I	1	EC
20	10 SH 559	69	I	2	EC
21	10 SH 724	72	I	1	EC
22	09 SH 645	56	I	2	EC
23	10 AH 600	76	I	3	EC
24	11 AH 257	78	I	1	EC
25	10 AH 347	58	I & II	2	EC, MMMT
26	10SH 582	74	I & II	3	EC, MMMT
27	08 SH 246	56	II		SPC
28	08 SH 304	73	II		SPC, CCEC
29	08 SH 565	86	II		MMMT
30	08 SH 937	83	II		MMMT

Primary human endometrial cancers samples in this study were collected from 24 type I cancer, 4 of type II cancer and 2 of mixture of type I and II cancer. EC: endometrioid adenocarcinoma; MMMT: mixed Mullerian malignant tumour; CCEC: clear cell endometrioid carcinoma; SPC: serous papillary carcinoma.

TABLE 5.2. Antibodies used for flow cytometry

Primary antibody	Clone name	Isotype control	Concentration
Anti-human CD24	ML5	mouse $IgG2_A$	1 µg/ml
Anti-human CD29	mAb13	rat $IgG2_A$	5 µg/ml
Anti-human CD49f	GoH3	rat $IgG2_A$	5 µg/ml
Anti-human CD44	G44-26	mouse $IgG2_B$	10 µg/ml
Anti-human CD45-PeCy5.5	unknown	mouse IgG_1	10 µg/ml
Anti-human CD10-PE	unknown	mouse IgG_1	10 µg/ml
Anti-human CD90-APC	5E10	mouse IgG_1	10 µg/ml
Anti-human CD31-PE	WM59	mouse IgG_1	10 µg/ml
Biotinylated anti-human EpCAM	BerEP4	biotinylated goat IgG	10 µg/ml
Secondary antibody			
Chicken-anti-rat IgG-A488		rat $IgG2_A$	5 µg/ml
Goat-anti-mouse $IgG2_B$-A488		mouse $IgG2_B$	5 µg/ml
Goat-anti-mouse $IgG2_A$-A647		mouse $IgG2_A$	5 µg/ml
Goat-anti-mouse IgG_1-PE		mouse IgG_1	1 µg/ml
Streptavidin-anti mouse IgG APC/A750		mouse IgG	1 µg/ml

PeCy5.5, phycoerythrin and cyanine 5.5; PE, phycoerythrin; APC, Allophycocyanin; A488, Alexa Fluor 488; A647, Alexa Fluor 647, APC/A750, Allophycocyanin and Alexa Fluor 750

5.2.4 Flow cytometry gating strategy

The electronic gating strategy for primary EnCa and EnCa cell lines was slightly different. Given that EnCa cell lines were immortal, of epithelial origin and have a normally distributed surface marker expression, only viable, live cells ($7ADD^-$ cells) were required to set the gating. This electronic gating will be illustrated using EpCAM and CD44 surface markers (Fig. 5.1). Viable endometrial cancer cell lines were selected based on their forward scatter (FSC) and side scatter (SSC) (R3, Fig 5.1A), followed by live cells, $7ADD^-$ cells (R2, Fig. 5.1B) selection. The dot plot was set to select only single cells and excluding doublets (R5, Fig. 5.1C). Once all the selection was made, the EpCAM/CD44 sub-populations can be sorted (Fig. 5.1D).

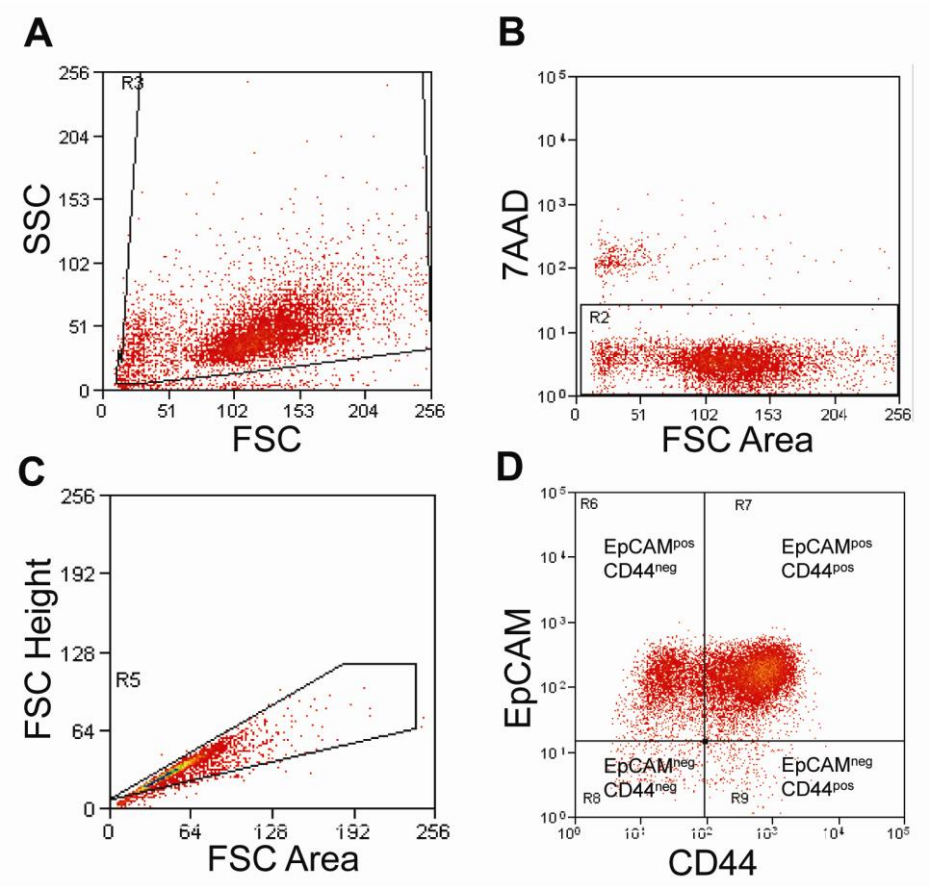


Figure 5.1 Electronic gating strategy for flow cytometry sorting of EnCa cell lines. A) Side scatter (SSC) and forward scatter (FSC) dot plot used to select viable cells (R3). **B)** Selected live cells (R2) and removed dead cells. **C)** Selected only single cells for sorting (R5), excluding dublets and cell clumps. **D)** A typical four-quadrant dotplot to select four different sub-populations of interest.

Primary EnCa cells contained the bulk of the tumour from an endometrial cancer tissue including many cell types and variation in surface marker expression, a more stringent electronic gating were required to eliminate stromal, leukocytes and endothelial cells and to set up appropriate negative controls for surface marker expression. This will be illustrated using CD44 and CD24 surface markers (Fig. 5.2). Primary EnCa cells were selected based on their forward (FSC) and side scatter (SSC) (R1, Fig. 5.2A) and eliminated $7AAD^{+}$ dead cells/debris (R11, Fig. 5.2B). Single cells (R10, Fig.5.2C) were selected, excluding stromal ($CD90^{+}$), leukocytes ($CD45^{+}$) and endothelial ($CD31^{+}$) cells

R2, Fig. 5.2D). Isotype-matched controls were used to set gates for CD44 and CD24 expression (Fig. 5.2E, F). Sub-populations of CD44/CD24 expressing cells can be sorted (Fig. 5.2G) into 4 sub-populations of interest and subjected to colony-forming assays.

5.2.5 Immunohistochemistry

OCT embedded type I endometrial cancer samples were cryosectioned (5 µm) and fixed in acetone for 2 mins. All sections were washed twice in PBS between each incubation steps unless stated otherwise. Sections were incubated with protein blocking solution (Dako) for 10 min. Sections were incubated with primary antibodies (diluted in 0.1 % BSA/PBS) against CD24, CD49f, CD29 and CD44 and their isotype-matched control, IgG as detailed in Table 5.2 for 1 hr at 37ºC. Sections were then incubated with biotinylated secondary antibody (Dako) for 15 min at room temperature (RT), followed by streptavidin-HRP (Dako) for another 15 min at RT. Staining was visualised by applying DAB chromogen (Sigma Aldrich) for 15 min at RT and counterstained with Meyer's haematoxylin (Amber Scientific, WA, Australia) and mounted in DPX and coverslips. Sections were analysed using Ziess AxioSkop microscope and image was acquired and using AxioCam camera and AxioVision software (version 4.6) (Gottingen, Germany).

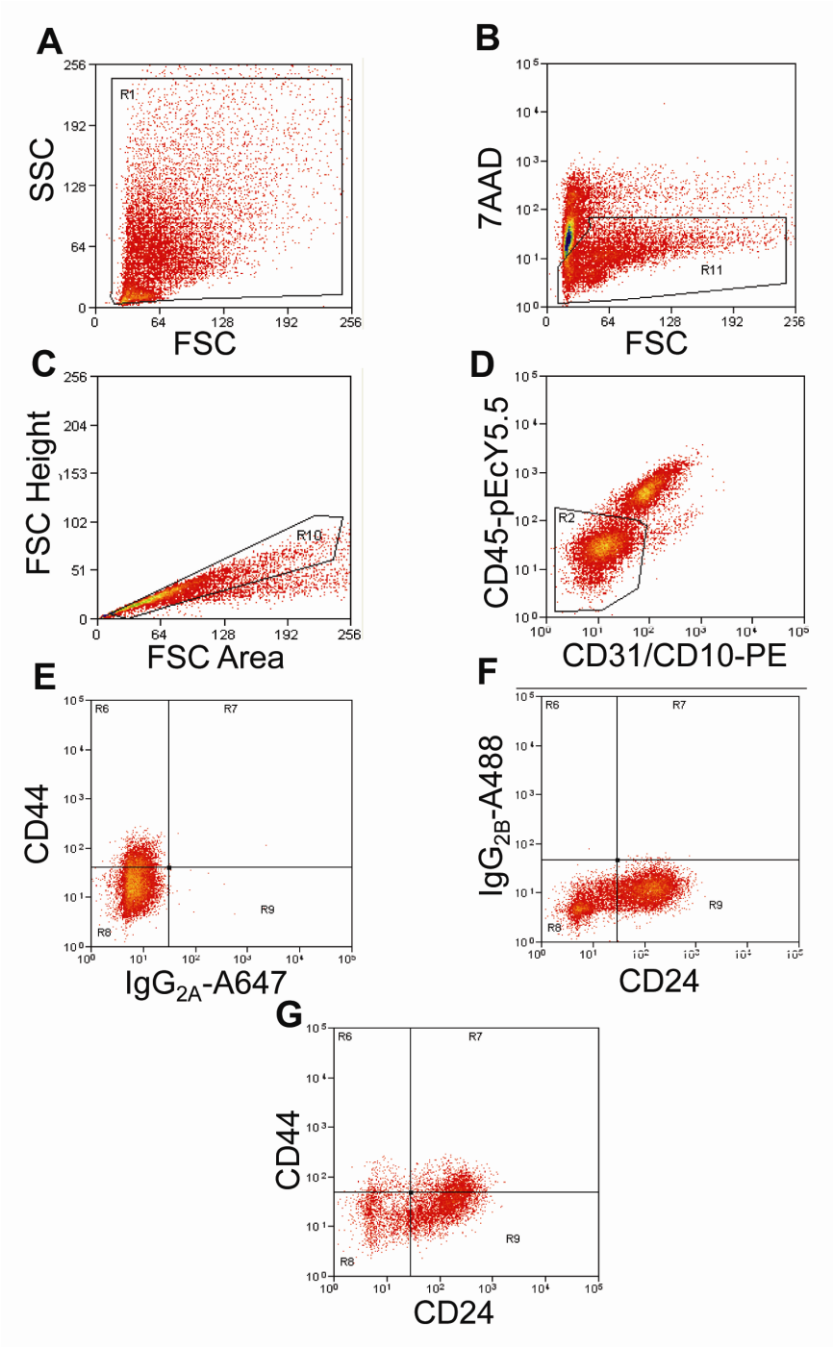


Figure 5.2 Electronic gating strategy for primary EnCa cells. A) Forward scatter (FSC) and side scatter (SSC) dot plot used to select viable cells. **B)** Select live cells (R11) and remove dead cells. **C)** Selection of single cells only for sorting (R10), excluding doublets and cell clumps. **D)** Gating to electronically exclude leukocytes ($CD45^+$) and stromal and endothelial cells ($CD10^+$), and selected R2 for sorting. **E)** Isotype-matched control (IgG_{2A}) on the x-axis is used to set vertical gate for CD24 expression. **F)** IgG_{2B} on the y-axis is used to set horizontal gate for CD44 expression. **G)** A typical four-quadrant dot plot to select for four different sub-populations of CD44/CD24 expression.

5.2.6 Stem cell functional assays

5.2.6.1 In vitro colony-forming ability of sorted endometrial cancer cells

Flow cytometry sorted sub-populations of EnCa cells and EnCa cell lines were seeded at clonal density (50 – 500 cells/cm^2 for primary EnCa and 20 – 100 cells/cm^2) as described previously (Chapter 4) with minor modifications. Primary EnCa cells required stromal feeder layer support for growth after sorting. Mitomycin-C inactivated human endometrial stromal cells (3000 cell/cm^2) (Chapter 2) was seeded onto fibronectin-coated (10 µg/ml) plates and allowed to adhere for at least 4 hr prior to seeding the sorted cells. Clonal cultures were incubated for 4 weeks and medium was changed every 6 – 7 days. Colony visualisation and cloning efficiency was performed as described in Chapter 4. Colony-forming activity was used as an initial screening assay for adult stem cell function of the sorted sub-populations. Due to the limited number of type II EnCa samples available for the study, only type I primary EnCa were used for immunohistochemistry and flow cytometry analysis and sorting. Due to time constraint of the study, flow cytometry sorting of Ishikawa cells were not investigated. All flow cytometry analysis and sorting of endometrial cancer cell lines were performed on 4 replicates using cells from 4 different passages, except for Ishikawa cell line (n = 1).

5.2.6.2 Sphere-forming culture of endometrial cancer cells

Single cell suspension of primary EnCa (Section 5.2.2), ECC1, Ishikawa and HEC-1A cells (1x10^6 cell/ml) were grown in NeuroCult medium (StemCell Technologies, in Ultralow attachment flasks (Corning, Corning Life Sciences, CA, USA) for 7 days to enable spheres to form, according to the manufacturer's instructions. Spheres were collected by centrifugation and pelleted and medium was changed every 2 days. For

propagation, spheres were digested enzymatically for 5 min using trypsin (Invitrogen) to a single cell suspension, counted and sphere formation was re-established by seeding at 2.5 $x10^4$ / 6 ml in a T25 non-adherent flask (StemCell Technologies). Spheres were allowed to grow until no further growth was observed or spheres were used for subsequent experiments.

5.2.7 Statistical analysis

All data were analysed using GraphPad Prism software (version 5.01, CA, USA). Flow cytometric data and colony-forming activity data are presented as mean ± standard deviation. Gaussian distribution was assessed using D'Agostino and Pearson omnibus normality test. Mann-Whitney U test was used for comparison between two groups. Two-way ANOVA or Kruskal-Wallis was used to compare three groups for parametric and non-parametric data respectively. Differences of $p < 0.05$ (95% confidence interval) were considered statistically significant.

5.3 Results

5.3.1 Colony-forming activity of endometrial cancer sorted cells

Four candidate markers, CD24, CD49f, CD29 and CD44 were examined on freshly isolated human primary endometrial cancer cells and endometrial cancer cell lines (ECC1, Ishikawa and HEC-1A) by immunohistochemistry and by flow cytometry using a multi-colour protocol. Given that there was no known single marker that can enrich for endometrial cancer stem cells, each marker was combined with EpCAM epithelial specific marker that is expressed by most cancers of epithelial origin (Litvinov *et al.*, 1994;

Baeuerle & Gires, 2007) and on endometrial epithelial cells (Chapter 2) to identify possible markers for the prospective isolation of endometrial cancer stem cells.

5.3.1.1 CD24 expression

CD24 strongly immunostained tumour cells in type I endometrial cancer (n = 4) (Fig. 5.3A), however, when examined by flow cytometric analysis, a great variation in expression was observed. CD24 expressed by 15.7 ± 11.4% (n = 4) of primary EnCa (Fig. 5.3B), but little to no expression by ECC1 (Fig. 5.3C), and 23.04 % (n = 1) by Ishikawa (Fig. 5.3D) and 67.1 ± 14.3% (n = 4 replicates) by HEC-1A cells (Fig. 5.3E). EpCAM/CD24 dual colour flow cytometry analysis was then examined on primary EnCa cells and the cells were sorted into 4 sub-populations (Fig. 5.3F and G). Due to the lack of CD24 expression, flow cytometry sorting of ECC1 cells for EpCAM/CD24 was not pursued. Sorted sub-populations of EpCAM/CD24 primary EnCa cells were examined for colony-forming activity, however, the cells did not attach and therefore no colonies were observed.

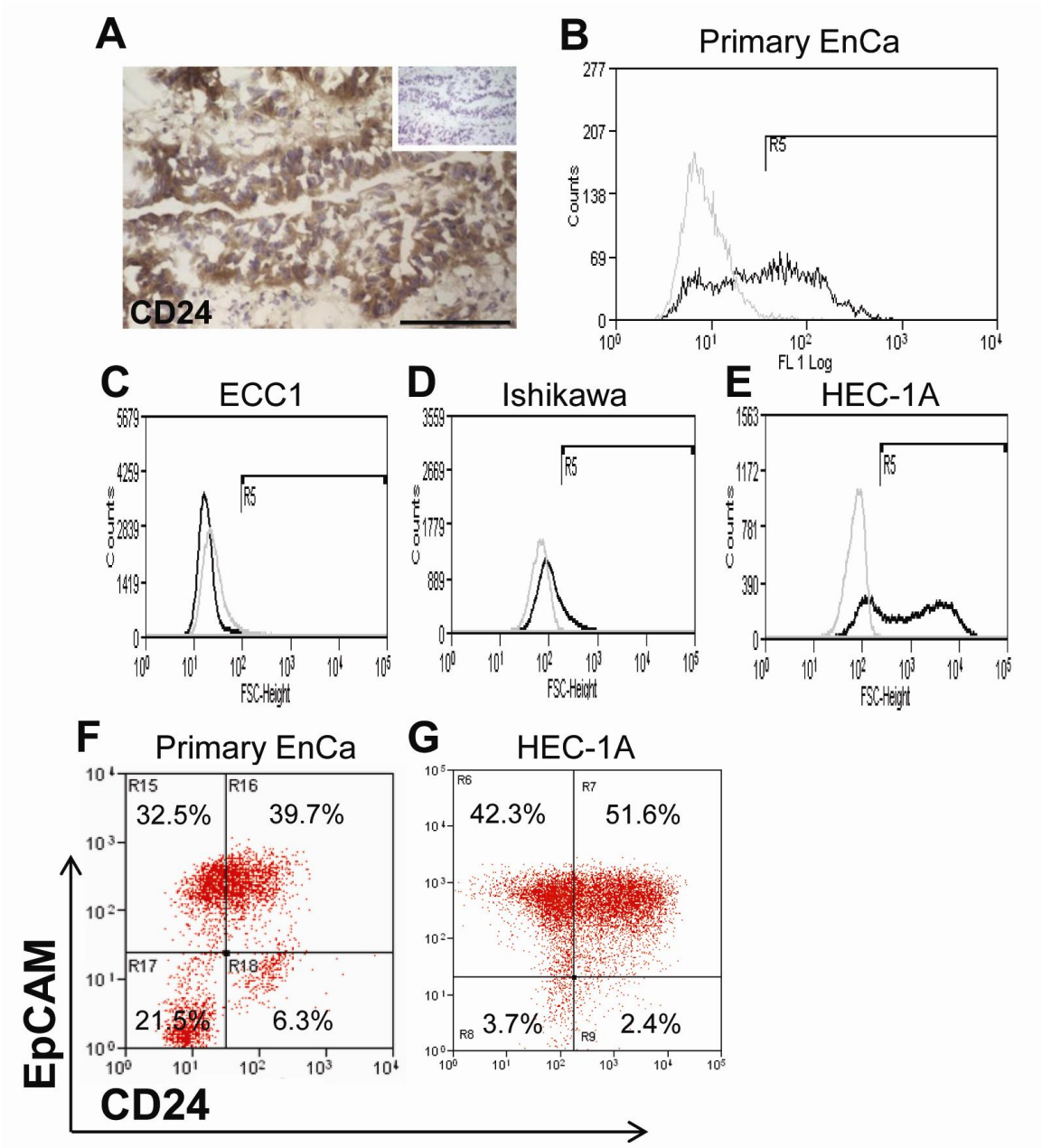


Figure 5.3 CD24 marker expression. A) CD24 immunostained primary EnCa glandular epithelial cells (n = 4). Insets: negative control. Single colour flow cytometry histograms showing CD24 expression in **B)** primary EnCa, **C)** ECC1 **D)** Ishikawa and **E)** HEC-1A cells. Dual colour flow cytometry dot plot of sorted EpCAM/CD24 sub-populations of **F)** primary EnCa and **G)** HEC-1A cells. Flow cytometry data are representative of n = 4, except for Ishikawa (n = 1).

5.3.1.2 CD49f expression

CD49f localised to the baso-lateral surface of adenocarcinoma cells of type I EnCa (n = 4) (Fig. 5.4A). CD49f expressed by 62.2 ± 7.2% of primary EnCa cells (n= 4) (Fig. 5.4B), 90.5 ± 5.5% (n = 4 replicates) of ECC1 cells (Fig. 5.4C), 98% of Ishikawa cells (Fig. 5.4D) and 92 ± 6.3% (n = 4 replicates) of HEC-1A cells (Fig. 5.4E). It appeared that CD49f expression is lowered in primary EnCa compared to cell lines, however majority of primary EnCa and EnCa cell lines expressed CD49f. EpCAM/CD49f dual colour flow cytometry analysis was analysed on primary EnCa, ECC1 and HEC-1A cells (Fig. 5.4F-H). When sorted EpCAM/CD49f sub-populations ($EpCAM^{neg}CD49f^{neg}$, $EpCAM^{neg}CD49f^{pos}$ and $EpCAM^{pos}CD49f^{pos}$) of primary EnCa cells were examined, there was one particular small subset within $EpCAM^{pos}CD49f^{pos}$ sub-populations of interest (R12, Fig. 5.4F). It distinct distribution from the main population, indicated it may be endometrial cancer stem cells, however, like other sub-populations of the primary EnCa cells, they failed to form colonies (data not shown) in the conditions used in this study. In contrast, the three sorted sub-populations ($EpCAM^{neg}CD49f^{neg}$, $EpCAM^{neg}CD49f^{pos}$ and $EpCAM^{pos}CD49f^{pos}$) of ECC1 cells initiated two types of colonies, large and small (Fig. 5.4I). However, the cloning efficiency was similar between the three sub-populations ($p = 0.8$). Interestingly, cloning efficiency was statistically significant between large and small clones of $EpCAM^{pos}CD49f^{pos}$ ($p = 0.02$) (Fig. 5.4J). Thus, $EpCAM^{pos}CD49f^{pos}$ could be a promising subset for further investigation.

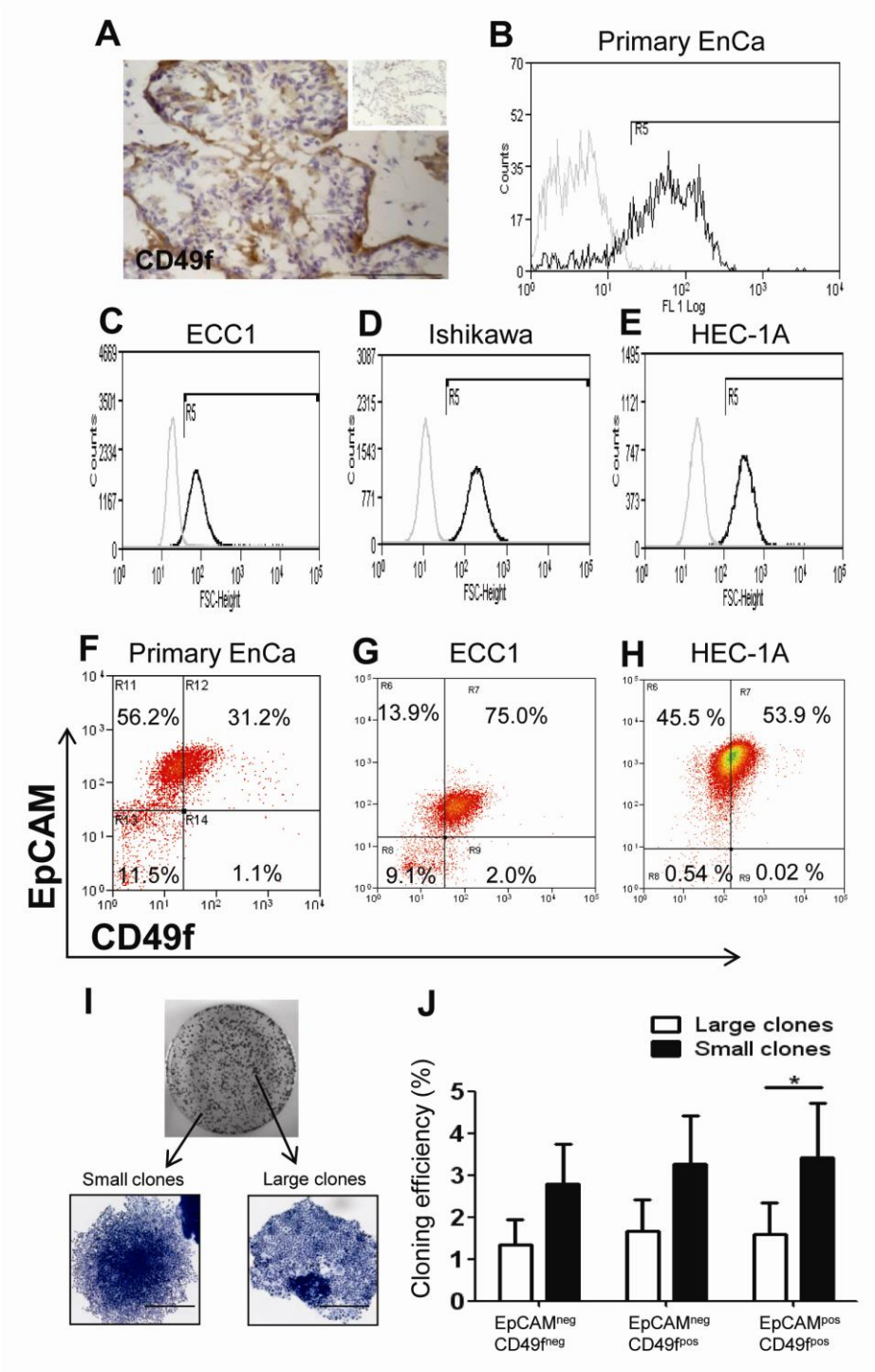


Figure 5.4 CD49f expression. A) Immunostaining of CD49f in type I EnCa (n = 4). Insets: negative control. Single colour flow cytometry histograms showing CD49f expression in **B)** primary EnCa, **C)** ECC1, **D)** Ishikawa and **E)** HEC-1A. Dual colour flow cytometry dot plot of EpCAM/CD49f sub-populations of **F)** primary EnCa, **G)** ECC1 and **H)** HEC-1A cells. **I)** A typical cloning plates of ECC1 sorted cells and colony morphological features of small and large clones. **J)** Colony-forming activity of three sorted EpCAM/CD49f sub-populations of ECC1 cells. Scale bar = 100 µm. Flow cytometry data are representative of n = 4, except for Ishikawa (n = 1).

5.3.1.3 CD29 expression

CD29 immunostained the majority of tumour cells of type I EnCa (n = 4) (Fig. 5.5A). However, flow cytometric analysis showed that CD29 was expressed on 43.6 ± 6.5% (n = 4) primary EnCa (Fig. 5.5B), indicating enzyme sensitivity. CD29 was also expressed on 90.8 ± 5.1% (n = 4 replicates) ECC1 cells (Fig. 5.5C), 98.4% Ishikawa cells (Fig. 5.5 D) and 97.7 ± 3.2% (n = 4 replicates) of HEC-1A cell (Fig. 5.5E). Similar to CD49f, CD29 expression appeared consistently lowered in primary EnCa compared to EnCa cell lines. The flow cytometry profile for CD29 and CD49f were similar between Ishikawa and HEC-1A.

EpCAM/CD29 dual colour flow cytometry analysis was analysed on primary EnCa, ECC1 and HEC-1A cells (Fig. 5.5F-H). Sorted sub-populations of EpCAM/CD29 ($EpCAM^{neg}CD29^{neg}$, $EpCAM^{neg}CD29^{pos}$ and $EpCAM^{pos}CD29^{pos}$) of primary EnCa did not form any colonies (data not shown). In contrast, all three sorted EpCAM/CD29 sub-populations of HEC-1A cells formed colonies. All colonies had the same morphological features (Fig. 5.5I) and there was no distinct small and large clonal morphology, which was as expected (Fig.5.5I). The cloning efficiency was similar between the sub-populations ($p = 0.7$) (Fig. 5.5J).

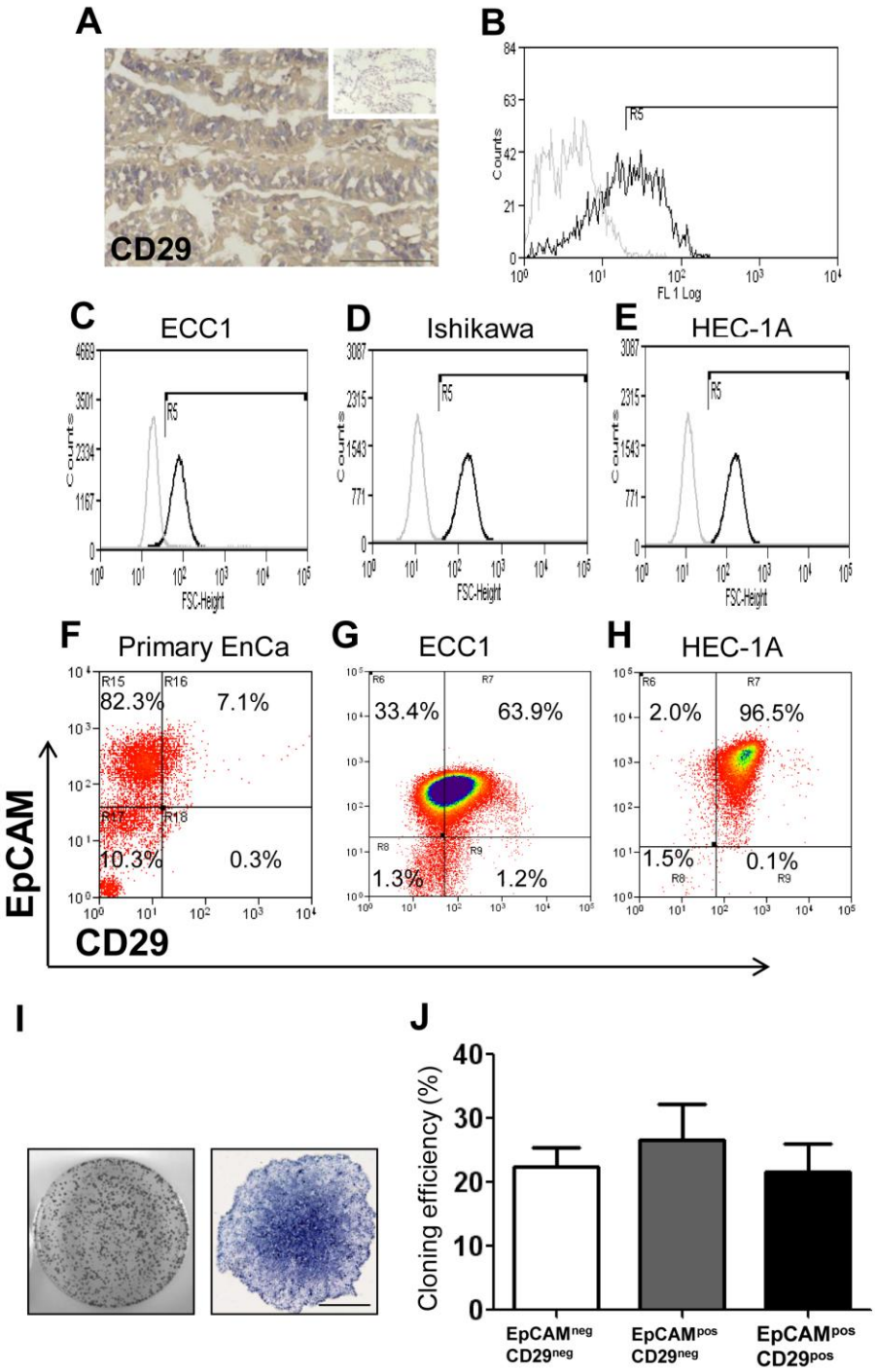


Figure 5.5 CD29 expression. A) Immunostaining of CD29 in type I EnCa (n = 4). Insets: negative control. Single colour flow cytometry histograms showing CD29 expression in **B)** primary EnCa, **C)** ECC1, **D)** Ishikawa and **E)** HEC-1A. Dual colour flow cytometry dot-plot of EpCAM/CD29 sub-populations of **F)** primary EnCa, **G)** ECC1 and **H)** HEC-1A cells. **I)** A typical cloning plates of HEC-1A sorted cells and colony morphological features. **J)** Colony-forming activity of three sorted EpCAM/CD29 sub-populations of HEC-1A cells. Scale bar = 100 µm. Flow cytometry data are representative of n = 4, except for Ishikawa (n = 1).

5.3.1.4 CD44 expression

CD44 showed strong immunoreactivity to tumour cells of type I EnCa (n = 4) (Fig. 5.6A). However, the flow cytometric analysis showed that CD44 was expressed on only 14.7 ± 6.9% (n = 4) of primary EnCa (Fig. 5.6B), indicating enzyme sensitivity, CD44 also expressed on 69.6 ± 11.3% (n = 4 replicates) of ECC1 cells (Fig. 5.6C), 98% of Ishikawa cells (Fig. 5.6D) and 80.3 ± 21.3% (n = 4 replicates) of HEC-1A cells (Fig. 5.6E). CD44 also co-expressed with EpCAM, however the flow cytometric profile was more similar between ECC1 and HEC-1A cell lines compared to primary EnCa, which show a small $CD44^{pos}EpCAM^{pos}$ population (Fig. 5.6F).

When EpCAM/CD44 sub-populations ($EpCAM^{neg}CD44^{neg}$, $EpCAM^{neg}CD44^{pos}$ and $EpCAM^{pos}CD44^{pos}$) (Fig. 5.7) of primary EnCa cells were examined, only $EpCAM^{pos}CD44^{pos}$ appeared to attach to cloning plates after 14 days in culture (Fig. 5.7C), however, no colonies were initiated. Similar to CD49f, three sorted EpCAM/CD44 sub-populations of ECC1 cells initiated two types of clones, large and small (Fig. 5.7A, D, E). The cloning efficiency was similar between the sub-populations (p = 0.3) (Fig. 5.7G), however, significant difference was observed between large and small clones of $EpCAM^{neg}CD44^{pos}$ subset (p = 0.001) (Fig. 5.7G). Interestingly, when HEC-1A cells were sorted for EpCAM/CD44, all four sub-populations ($EpCAM^{neg}CD44^{neg}$, $EpCAM^{neg}CD44^{pos}$, $EpCAM^{pos}CD44^{pos}$ and $EpCAM^{pos}CD44^{neg}$) initiated and formed clones at a similar frequency. There was only one type of clone (Fig. 5.7B, F) and cloning efficiency was similar between the sub-populations (p = 0.3). As expected, there was no significant difference when colony-forming activity of EpCAM/CD44 sub-populations of ECC1 and HEC-1A cells were compared (p = 0.3) (Fig. 5.7H).

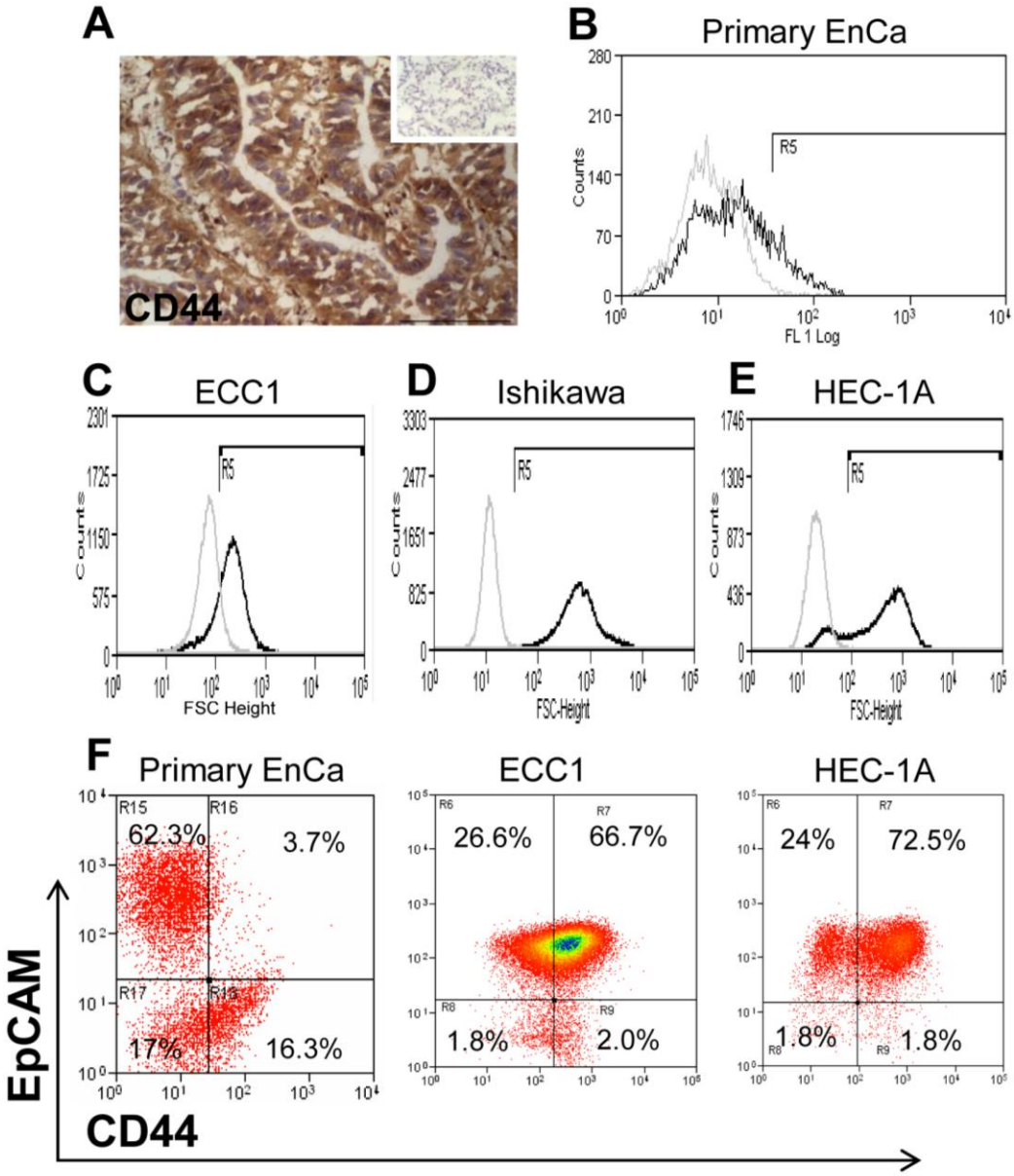


Figure 5.6 CD44 expression. A) Immunostaining of CD44 in type I EnCa (n = 4). Insets: negative control. Single colour flow cytometry histograms showing CD44 expression in **B)** primary EnCa, **C)** ECC1, **D)** Ishikawa and **E)** HEC-1A cells. **F)** Dual colour flow cytometry dot plot of EpCAM/CD44 sub-populations of primary EnCa, ECC1 and HEC-1A cells respectively. Flow cytometry data are representative of n = 4, except for Ishikawa (n = 1).

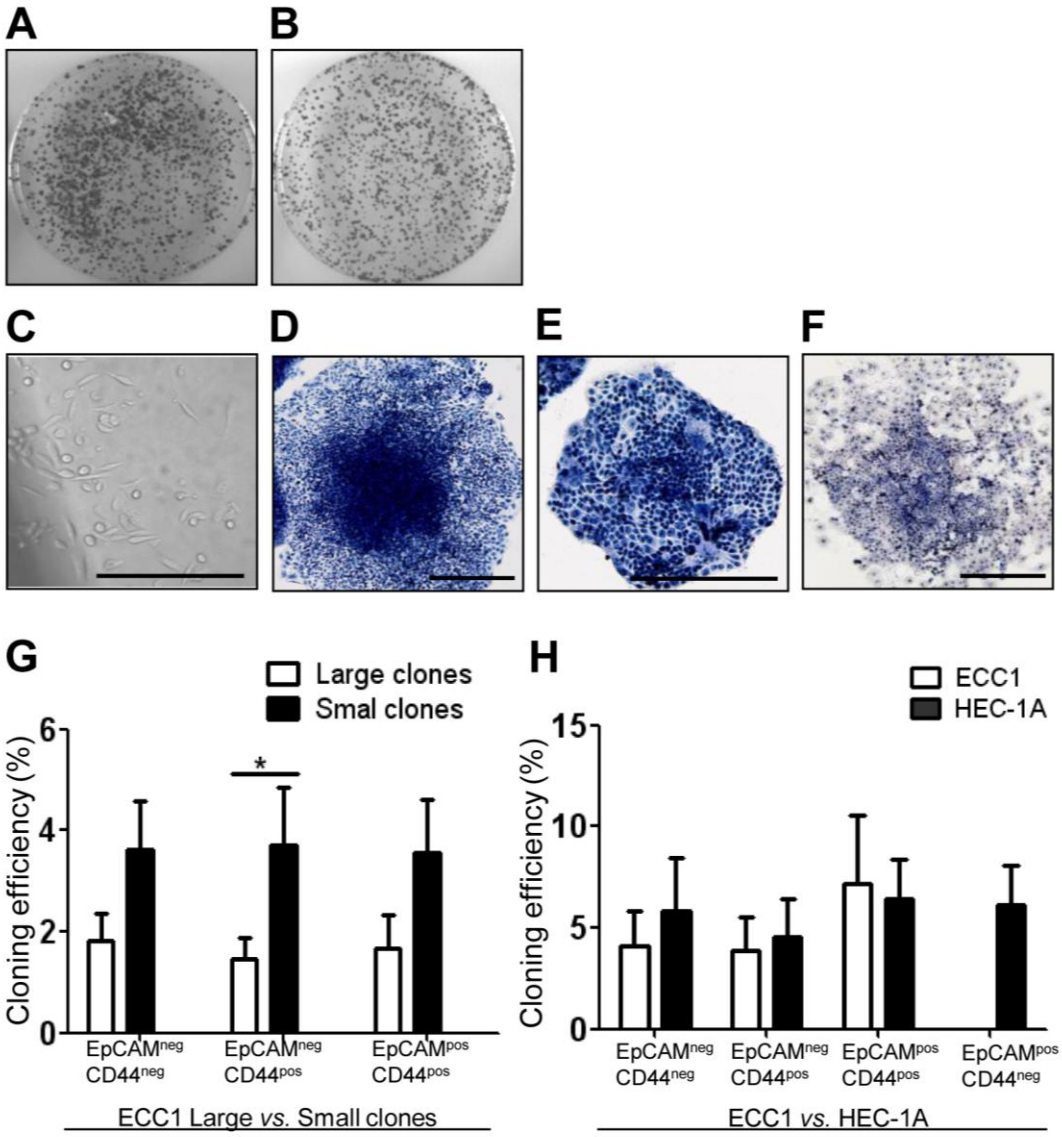


Figure 5.7 Colony-forming activity of sorted EpCAM/CD44 sub-populations of EnCa cells. A typical cloning plates of **A)** ECC1 and **B)** HEC-1A sorted cells. Morphological features of EpCAM/CD44 sorted cells of: **C)** Primary EnCa cells after 14 days in culture. **D)** Large clones and **E)** Small clones of ECC1 sorted cells. **F)** HEC-1A sorted cells. **G)** Comparison of colony-forming (CFU) activity between large clones (white bars) and small clones (black bars) of three sorted EpCAM/CD44 sub-populations of ECC1 cells. **H)** Comparison of CFU activity between four sorted EpCAM/CD44 sub-populations of ECC1 (white bars) and HEC-1A (black bars) cells. No distinction was made between large and small clones in HEC-1A cells. Scale bar = 5 µm.

Regardless of surface markers examined, none of the sorted sub-populations of primary EnCa cells initiated and formed colonies, indicating that primary EnCa cells could be sensitive to the harsh flow cytometry sorting process and may need some support for clonal growth. Since sorted endometrial epithelial cells required feeder layers (Tan, 2011) (Chapter 2), it was decided that stromal feeder should be used to provide culture support for primary EnCa sorted sub-populations (Fig. 5.8A). In this study, we chose to investigate co-expression of CD44/CD24 as this combination of markers was used to enrich for breast cancer stem cells (Al-Hajj *et al.*, 2003) and the CD44 marker has been used widely to enrich for stem cells in other solid cancers (Dalerba *et al.*, 2007; Li *et al.*, 2007; Prince *et al.*, 2007; Zhang, 2008).

Four sub-populations of primary EnCa cells (n = 4) were sorted ($CD44^{neg}CD24^{neg}$, $CD44^{neg}CD24^{pos}$, $CD44^{pos}CD24^{neg}$ and $CD44^{pos}CD24^{pos}$) and colony-forming activity of each sub-population cultured with and without stromal feeder layer (Fig. 5.8 B, C). As expected, the sorted cells grew better with the stromal feeder layer and had higher cloning efficiency (Fig. 5.8D). Although there was no significant difference in cloning efficiency between sub-populations ($p = 0.9$), it appeared that $CD44^{pos}CD24^{neg}$ sub-population had the highest cloning efficiency compared to other subsets.

5.3.2 Sphere formation ability of endometrial cancer cells

The initial colony-forming activity screening of the four candidate markers (CD24, CD49f, CD29 and CD44), provided no clear indication of which marker could be used to enrich for endometrial cancer stem cells. It was decided to investigate the sphere formation of primary EnCa cells and EnCa cell lines. Both ECC1 and HEC-1A cell lines formed spheres after 7 days in culture and propagated as secondary and tertiary spheres (Fig. 5.9A, B).

Type II primary EnCa cells also formed spheres and propagated secondary and tertiary spheres, however type I EnCa cells were incapable of anchorage independent growth and sphere formation (Fig. 5.9C). Paradoxically, the type I cell line, ECC1 generated spheres which were larger and denser than type II primary EnCa and HEC-1A cell lines. ECC1 cell lines also propagate more readily compared to HEC-1A and primary type II EnCa cells. Spheres also expressed candidate stem cell surface markers, CD24 (87.9%), CD49f (7.8%), CD29 (7.6%) and CD44 (31%), with variation in the level of expression (Fig. 5.9D). Spheres also expressed 50% of EpCAM and were negative for stromal marker (CD10).

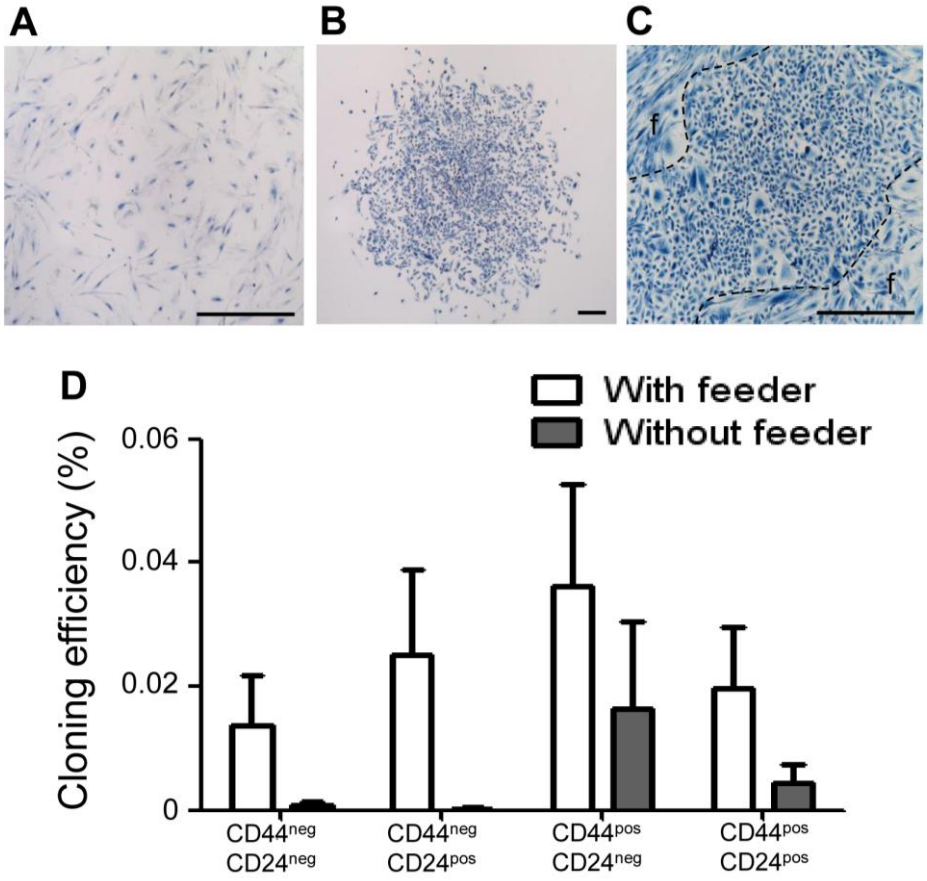


Figure 5.8 Colony-forming activity of CD44/CD24 human endometrial cancer sub-populations grown with and without endometrial stromal feeder layer. A) A typical feature of endometrial stromal feeder. **B & C)** Morphology of clones grown without and with stromal feeder respectively. **D)** Cloning efficiency of sorted sub-populations grown with and without stromal feeder layer. f; stromal feeder layer. Scale bar = 50 µm. Data are presented as mean ± standard deviation and representative of n = 4 primary EnCa samples.

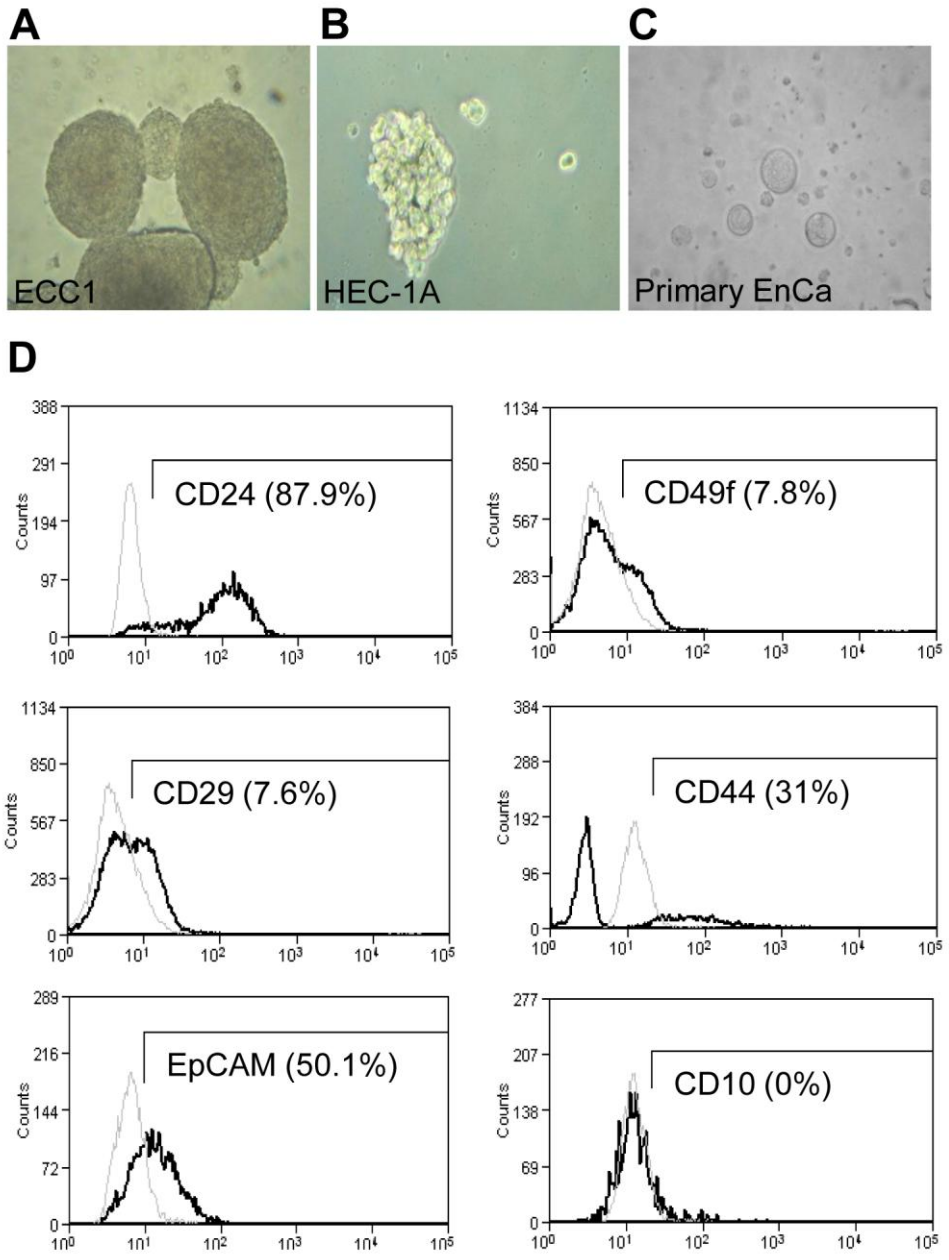


Figure 5.9 Sphere formation of endometrial cancer cells. A-C) A typical features of spheres generated by ECC1, HEC-1A and type II primary EnCa cells. **D)** Surface marker expression of sphere-forming cells, showing great level of variation. Data was from one individual patient sample.

5.4 Discussion

This study demonstrated that primary EnCa and cell lines (ECC1, Ishikawa and HEC-1A) expressed candidate stem cells markers CD24, CD49f, CD29 and CD44 which identify CSC in tumour of other organs. In general, the percentage of positive cells for the surface markers was similar between different EnCa cell lines. This was evident by the similarity in the percentage of positive cells observed for CD49f, CD29 and CD44 between type I cell lines, ECC1 and Ishikawa and type II cell line, HEC-1A. Surface marker expression was lowered in type I primary EnCa compared to the cell lines. Combinations of sorted sub-populations (EpCAM/CD49f, EpCAM/CD29 and EpCAM/CD44) of ECC1 and HEC-1A cell lines but not primary EnCa exhibited clonogenic activity. However, all subsets had similar cloning efficiency, indicating that these markers do not enrich for a CSC population on the basis of clonogenicity. Regardless of surface markers, ECC1 sorted cells gave rise to two types of clones, small and large, while HEC-1A sorted cells only initiated one type of clone. This study also showed that only type II primary EnCa and cell lines formed spheres expressing candidate stem cell surface markers. Another important finding of this study was that endometrial stromal feeder layer was essential for the clonal cultures of sorted primary EnCa cells.

CD44, an adhesion molecule well known for regulating cell proliferation, migration and invasion, and is frequently used to isolate cancer stem cells. CD24 is another adhesion molecule, a stem cell marker (Gao *et al.,* 2010) when combined with CD44 has proven to be a valuable set of markers to isolate cancer stem cells from tumours of several organs. In fact, the first cancer stem cells to be identified in solid cancers used $CD44^{pos}CD24^{neg}$ (Al-Hajj *et al.,* 2003). CD44/CD24 sub-populations of sorted primary EnCa in this study formed colonies, and interestingly, $CD44^{pos}CD24^{neg}$ had the highest cloning efficiency,

although not significantly different from other sub-populations. This finding is potentially significant in that both the breast and the endometrium are hormonally regulated (Jabbour *et al.*, 2006; Asselin-Labat *et al.*, 2010). It suggests that $CD44^{pos}CD24^{neg}$ may be potential candidates to enrich for endometrial cancer stem cells. However, more samples are required to confirm this preliminary finding. Although, CD44 is expressed in many isoforms due to alternative splicing, in this study, the antibody used (clone G44-26) has been shown to recognise all CD44 isoforms and had the ability to isolate CSC of the breast, pancreas and colon (Dalerba *et al.*, 2007; Orian-Rousseau, 2010; Olsson *et al.*, 2011). Similarly, it was expected that it could be used to isolate endometrial cells with clonogenic activity.

In addition, the cloning efficiency of $CD44^{pos}CD24^{neg}$ increased with stromal feeder support, highlighting the importance of epithelial-stromal interactions in the reproductive systems (Cunha & Lung, 1979; Kurita *et al.*, 2001). It is likely that the stromal cells may be acting as cancer stem cells niche (Kalluri & Zeisberg, 2006; Li & Neaves, 2006; Taylor & Risbridger, 2008) providing paracrine estrogen receptor signalling known to promote endometrial cancer growth (Matsumoto *et al.*, 2008). Although there was no significant, a greater number of clones were generated by EpCAM/CD44 sub-populations of ECC1 and HEC-1A sorted cells, suggesting the usefulness of CD44 as a potential marker. Presently, preliminary evidence shown in this study indicates that CD44 may be used to purify and enrich for endometrial cancer stem cells and is worth pursuing in further studies using larger number of samples and wider range of assays, particularly *in vivo* stem cell functional assays.

Colony-forming ability is the simplest and most common *in vitro* screening assay of adult stem cell activity that can be used in prioritising candidate markers (Schwab, 2008;

Hubbard & Gargett, 2010). The frequency of colony-forming endometrial cancer cells reported in this study is comparable with that of normal endometrial epithelial cells (Chan *et al.*, 2004) and unfractionated endometrial cancer cells (Hubbard *et al.*, 2009). Similar frequencies of colony-forming activity have been observed for other cancers including the brain, colon and retinoblastoma (Singh *et al.*, 2003; Dalerba *et al.*, 2007; Zhong *et al.*, 2007).

Different sized clones were observed in sorted ECC1 cells suggesting that multiple subsets, including the more differentiated progenitor cells likely exist in this cell line and contribute to the cloning frequency (Hubbard *et al.*, 2009). Consistent with this study was the finding that different sized clones were observed in primary type I EnCa and not in type II EnCa (Hubbard *et al.*, 2009). A trend observed in this study was that sorted type II cell line HEC-1A had higher cloning efficiency compare to that of type I cell line ECC1. However, examining cloning activity alone is not enough to draw a conclusion whether HEC-1A comprise CSC or to prioritise surface marker candidate. It is possible that the clones generated from HEC-1A are from the progenitor cells that adapted to long term culture conditions and propagation. Thus *in vivo* tumourigenicity is essential to confirm and extend this finding. Based on data from a primary EnCa study, type II EnCa is more aggressive and can form tumours more readily compared to type I EnCa, thus one would expect HEC-1A cells to have similar tumour forming ability as primary type II EnCa cells. Additionally, marker expression in endometrial cancer cell lines was not able to delineate a separate endometrial cancer stem cell population. Together, this data suggests that other stem cell functional assays should be investigated. Nevertheless, it does indicates that a small, rare population of cells may be endometrial cancer stem cells and supports the hypothesis of their existence (Hubbard *et al.*, 2009).

Sphere formation and propagation of primary EnCa and ECC1 and HEC-1A observed in this study further supports the existence of rare endometrial cancer stem cells in both primary tumours and in cell lines. The frequency of sphere-forming was also consistent with reports in endometrial cancer (Rutella *et al.*, 2009) and in other systems (Cariati *et al.*, 2008). This approach should be used to investigate sorted CD44/CD24 sub-populations of EnCa cells in future experiments. There is some controversy on the validity of using sphere assays to accurately assess rare cancer stem cell activity, because spheres are unlikely to originate from a single cell but rather from aggregates of many cells (Reynolds & Rietze, 2005; Singec *et al.*, 2006; Visvader & Lindeman, 2008). However, studies have shown that sphere-forming cells grown in no-adherent manner retain and have a more similar molecular and histological phenotype to the parent tumour than cells that grown in monolayer (Zietarska *et al.*, 2007; Grun *et al.*, 2009). This is demonstrated by studies of neurospheres derived from highly malignant brain tumour where glioma cells formed tumour *in vivo* and recapitulating morphology of the parent tumour (Galli *et al.*, 2004; Laks *et al.*, 2009). However, in our study, only 50% of sphere-forming cells expressed EpCAM marker, suggesting that marker expression is altered in long term culture *in vitro* (Zaitseva *et al.*, 2006; Daniel *et al.*, 2009). Taken together, in the context of identifying cancer stem-like cell population, it is necessary to use multiple approaches to provide conclusive evidence of endometrial cancer stem cell enrichment.

In this study, we relied on the use of EnCa cell lines to optimise and prioritise for candidate markers that may enrich for endometrial cancer stem cells. This approach has been used successfully for identifying cancer stem cells in other organs (Cariati *et al.*, 2008; Fillmore & Kuperwasser, 2008; Chu *et al.*, 2009; Yeung *et al.*, 2010) however, they did not enrich for endometrial cancer stem cells with clonogenic activity, suggesting that the

immortalised cell lines contain a homogenous population of cells expressing most candidate stem cell markers. Consequently, this approach using ECC1, Ishikawa and HEC-1A have limited value for investigating candidate markers for the prospective isolation of endometrial cancer stem cells. Alternatively, this also indicates that multiple cancer-initiating cells may exist within endometrial cancer cell lines, all contributing to clonogenic activity. This present finding indicates that *in vivo* tumour initiation of different sorted sub-populations of EnCa cell lines is necessary before a definitive conclusion could be given.

Sorted sub-populations of EnCa cell lines survived, were superior in forming colonies after flow cytometry sorting compared to primary EnCa cells. This indicated that freshly isolated primary EnCa cells are very sensitive to the harsh process of flow cytometry sorting and that the sorting process greatly impacted on cell survival. Cell lines are adapted to cell culture, while freshly isolated EnCa cells are further compromised after sorting as they have to adapt to cell culture. This finding is in line with work from our laboratory and that of others (Laks *et al.*, 2009; Masuda *et al.*, 2011; Tan, 2011). To address this problem, endometrial stromal feeder support was used to help improve colony-forming activity of sorted cells and to our expectation, higher cloning efficiency was observed. This data may indicates that epithelial-stromal interactions is essential and that *in vitro* cultures could be lacking factors of the niche environment that is important for cell growth and proliferation (Fuchs *et al.*, 2004; Li & Neaves, 2006; Taylor & Risbridger, 2008).

During the course of this study, several groups published on potential markers such as CD133 and Musashi-1 as candidates of endometrial cancer stem cells (Götte *et al.*, 2008; Rutella *et al.*, 2009). In one study, $CD133^{+}$ cells were shown to have high cloning efficiency, proliferative potential, formed spheres, and were chemoresistance (Rutella *et*

al., 2009). However, recent work from our laboratory demonstrated that CD133 did not enrich for endometrial cancer cells with stem cells properties when examined *in vitro* and *in vivo* (Hubbard, 2010). These contradictory findings may explain the hypothesis that cancer stem cells may not be rare and that multiple cancer cell subsets may initiate and sustain the bulk of the tumour (Visvader & Lindeman, 2008). Furthermore, it could also be that different techniques were use for the study (Hubbard, 2010). Further investigation is necessary to confirm whether CD133 enriches for endometrial cancer stem cells.

In another study, Musashi-1, an RNA-binding protein associated with neural and intestinal stem cell (Pilkington, 2005; Sureban *et al.*, 2008) immunolocalised small clusters of endometrial cancer cells (Götte *et al.*, 2008). It was hypothesised that Musashi-1 expressing cells were endometrial progenitor or cancer stem cells. However, stem cell activity of Musashi-1 expressing cells was not investigated and until functional studies are undertaken, it is not known if Musashi-1 enriches for endometrial CSC.

Side population (SP) was another approach frequently used to identify cells enriched for stem cell and cancer stem cell activity. Using this approach, a side population have been identified in endometrial cancer and cell lines (Friel *et al.*, 2008; Kato *et al.*, 2010). The SP cells in these studies exhibited several cancer stem cell traits including self-renewal, high proliferative potential, chemoresistance and the ability to initiate tumours *in vivo* (Friel *et al.*, 2008; Teixeira *et al.*, 2008; Hubbard & Gargett, 2010; Kato *et al.*, 2010). Taken together, these findings provide conclusive evidence supporting the presence of rare cancer stem cells in endometrial cancer. However, it is unknown if endometrial cancer SP cells express CD133 or Musashi-1.

One of the limitations of this study was that the histo-pathological reports for samples were not available at the time of tissue collection and experimentation. According to the ethical approval and the tissue collection procedure set out by the hospital and the Victorian Cancer Biobank, the researchers are only provided with the diagnosis. Information including the cancer type, grade and stage was only available when the final report is written by the pathologist. This made it impossible to investigate all types and grades of EnCa samples for candidate markers. The availability of tissue samples dwindled during my candidature, limiting the investigation of clonogenicity of type II EnCa and sphere formation in type I EnCa. Another constraint of this study was that only 2-3 cancer cell lines were examined. Since other research groups have found side population with clonogenic activity, self-renewal and tumourigenicity in different EnCa cell lines (Friel *et al.*, 2008; Kato *et al.*, 2010), it may be worth examining these properties together with surface markers in future studies, or examining sphere formation and clonogenicity in different cell lines. In addition, given that magnetic bead sorting was used to successfully sort for potential candidate endometrial epithelial stem/progenitor cells (Chapter 4), it could be used in the future to sort primary EnCa cells and improve cell viability. Nevertheless, this study is the first to screen and compare the expression of a panel of known cancer stem cell markers in primary EnCa and EnCa cell lines ECC1, Ishikawa and HEC-1A.

The data presented in this study indicated that surface marker expression was more similar between cell lines than primary EnCa. All EnCa cell lines examined had colony-forming activity and sphere formation. We have confirmed previous work that flow cytometry sorting is very harsh and negatively affects survival and growth of primary EnCa cells. Stromal feeder layer support greatly enhanced cell growth and colony formation. Evidence

in this study shows a promising trend for $CD44^{pos}CD24^{neg}$ marker combination as a candidate to enrich for endometrial cancer stem cells. *In vivo* tumour initiation is essential to extend this preliminary finding. The prospective isolation of endometrial cancer cells will allow their characterisation and their role in endometrial cancer to be investigated.

Chapter 6

General discussion and future directions

This dissertation provides the first evidence confirming the likely location of epithelial stem/progenitor cells in the basalis layer of the endometrium. Prior to the commencement of this study, the origin and location of endometrial epithelial stem/progenitor cells were unknown. Rare clonogenic epithelial cells had been identified in pre- and post-menopausal endometrium from hysterectomy samples, suggesting their residence in a stem cell niche in the basalis and their possible role in endometrial regeneration. The data presented in this study is the first to provide evidence showing a different gene signature between pre-menopausal and basalis-like post-menopausal endometrial epithelial cell compartment. In addition, comparative analysis of this study and that of previous publication revealed similarities between post-menopausal endometrium and the basalis of menstrual endometrium.

N-cadherin, a surface marker identified from the differential gene list shows promise as a potential marker of human endometrial epithelial stem/progenitor cells. A small population of endometrial epithelial cells expressed N-cadherin and these were predominantly located in the basalis of pre-menopausal and post-menopausal endometrial glands. Sorted N-cadherin^{pos} endometrial epithelial cells were enriched for colony forming cells, which exhibited the stem cell property of self-renewal.

Known cancer stem cell markers (CD24 CD49f, CD29 and CD44) were examined for the first time in endometrial cancer and endometrial cancer cell lines (ECC1, Ishikawa and HEC-1A) as potential endometrial cancer stem cell markers. Surface marker expression showed variation between primary EnCa and cell lines. All sorted sub-populations of ECC1 and HEC-1A cells but not type I primary endometrial cancer cells had colony-forming activity. ECC1, HEC-1A and type II primary endometrial cancer cells had the ability to initiate sphere formation.

This final chapter will discuss the implications of identifying candidate markers of epithelial stem/progenitor cells on human endometrial epithelial cells and endometrial cancer and suggest areas for further research.

6.1 Isolation, culture and characterisation of human endometrial epithelial cells

Flow cytometry sorting is a powerful tool used for selecting purified epithelial stem cells from other organ systems on the basis of surface marker expression. However, this technique has not been applied to isolate endometrial epithelial stem cell sub-populations, due to lack of specific surface markers and the difficulty in maintaining long term cultures *in vitro*. Previously, published methods have outlined the use of enzymes and differential filtration to obtain glandular epithelium and established their growth in cultures (Kirk *et al.*, 1978; Satyaswaroop *et al.*, 1979). However, epithelial cell viability was greatly affected by the initial enzyme dissociation procedure, resulting in short survival times in cultures (Osteen *et al.*, 1989; Fernandez-Shaw *et al.*, 1992). This study refined several isolation protocols to produce a robust technique for obtaining endometrial epithelial single cell suspensions. Appropriate exposure to a combination of DNase 1, collagenase I and collagenase II enzymes allowed clear separation of endometrial epithelial glands and subsequent dissociation to single cells without compromising cell viability.

Flow cytometry was used to acquire and sort endometrial epithelial cells on the basis of EpCAM marker expression, which is expressed on virtually all endometrial epithelial cells (Chan *et al.*, 2004; Tan, 2011). It was necessary to consider the choice of marker and their respective fluorochomes to ensure a true positive epithelial cell surface marker expression for the study. While EpCAM selected endometrial epithelial cells with at least 99% purity,

an additional marker, CD90 was used to eliminate any contamination stromal cells remaining after the initial enzyme dissociation. This is important as adult stem cell populations typically comprise approximately 1% of the total cell population. This study also outlined a guide for careful compensation of fluorescence spectral overlap of fluorochromes, and to minimise cell death during lengthy cell isolation and sorting procedures. Although flow cytometry sorting isolated a homogenous endometrial epithelial cell population, the long duration unavoidably resulted in cellular damage, limiting epithelial cells from establishing clonal cultures.

Stromal cell feeder layers overcame the limitations of endometrial epithelial clonal culture of sorted cells (Chapter 2), similar to clonal culture of these cells in serum-free medium (Chan *et al.*, 2004). An alternative approach to flow cytometry sorting is magnetic bead sorting, a much simpler, gentler and shorter isolation procedure. It improved cell survival and increased clonogenic activity (Masuda *et al.*, 2011). Adopting these approaches enabled the isolation of highly purified EpCAM positive epithelial cells for the microarray study (Chapter3) and N-cadherin enriched endometrial epithelial cells with clonogenic activity (Chapter 4). Thus, the groundwork undertaken in Chapter 2 refining the endometrial epithelial cell isolation procedures to obtain a robust protocol was essential to for the work in subsequent Chapters. Further, it is essential for future characterisation of endometrial epithelial stem/progenitor cells and study of their unique biological properties.

6.2 Differential expression of Wnt signalling molecules between pre-and post-menopausal endometrial epithelial cells suggests a population of putative epithelial stem/progenitor cells reside in the basalis layer

It has been hypothesised that stem/progenitor cells reside in the basalis and that they are responsible for endometrial regeneration (Padykula *et al.*, 1989; Padykula, 1991; Gargett, 2007). Subsequent studies have identified clonogenic epithelial cells in pre- and post-menopausal endometrium (Chan *et al.*, 2004; Schwab *et al.*, 2005). Epithelial cell proliferation rates are also lower in the basalis of pre-menopausal and in post-menopausal endometrium compared to functionalis of pre-menopausal endometrium (Ferenczy *et al.*, 1979; Brenner *et al.*, 2003). Based on this evidence, and the gene profiling study of endometrial epithelial cells in Chapter 3, this study suggested that post-menopausal endometrium is similar to the basalis of pre-menopausal. Comparison with the gene profile of laser captured epithelium from the basalis of menstrual endometrium (Gaide Chevronnay *et al.*, 2009; Gaide Chevronnay *et al.*, 2010) verified a common gene signature. While numerous transcriptional profiling studies have identified differentially expressed genes across the menstrual cycle (Ponnampalam *et al.*, 2004; Talbi *et al.*, 2006) and in post-menopausal endometrium exposed to hormonal treatments (Klaassens *et al.*, 2006; Hanifi-Moghaddam *et al.*, 2007), this thesis provides the first gene profiling comparison between homogenous populations of epithelial cells isolated from pre-and post-menopausal human endometrium.

Many genes associated with the Wnt signalling pathway were differentially expressed between pre-and post-menopausal endometrial epithelial cells. This is of interest given that the Wnt signalling pathway is important in developmental processes and stem cell biology.

Nuclear localisation of Axin2, an inhibitor of Wnt signalling in epithelial cells suggests regulation of β-catenin transcriptional activity and maintenance of epithelial stem/progenitor cell quiescence in the basalis of pre-menopausal and in post-menopausal endometrium. Small molecules can be used to modulate Axin2 activity (Chen *et al.*, 2009; Huang *et al.*, 2009) to influence endometrial gland regeneration in each menstrual cycle. It is possible that Axin2 modulators are activated in post-menopausal endometrial epithelial cells when estrogen is administered to effect regeneration. This warrants further investigation, as there is very little research into the role of Axin2 in post-menopausal endometrium.

The Wnt signalling pathway is important in endometrial function and is involved in stem cell fate determination (Logan & Nusse, 2004; Reya & Clevers, 2005) and regulated by hormones (Kao *et al.*, 2002; Tulac *et al.*, 2003; Tulac *et al.*, 2006). Differentially expressed Wnt signalling and other stem cell networks identified in this study provide evidence of the likely existence of epithelial cells stem/progenitor cells and their niche in the basalis. To confirm the role of the Wnt signalling in endometrial regeneration, future studies could examine post-menopausal endometrium from women treated with hormone therapy, to investigate whether Wnt activates downstream target genes involved in reconstituting endometrial epithelium.

The highly up-regulated genes identified in post-menopausal endometrium (Chapter 3) were comparable to that of basalis epithelium of another study on menstrual endometrium, also a time when hormonal levels are very low (Gaide Chevronnay *et al.*, 2009; Gaide Chevronnay *et al.*, 2010). This comparative analysis highlights different gene contribution by the epithelial cells in the functionalis and basalis of pre-menopausal endometrium. Together, the data of this study and that of others provide evidence that post-menopausal

endometrium is similar to the basalis of pre-menopausal endometrium. It is possible that the basalis genes contribute to endometrial regeneration via regulating the stem cell compartment and while genes from the functionalis may be involved in endometrial breakdown and shedding. This study lays the groundwork enabling further investigation to identify markers of endometrial epithelial stem/progenitor cells and hence their role in endometrial regeneration.

6.3 N-cadherin isolates putative human endometrial epithelial stem/progenitor cells

Chapter 4 identifies the first candidate marker of endometrial epithelial stem/progenitor cells using a gene transcriptional profiling approach. The data generated in Chapter 3 enabled a short-listing of potential surface marker genes overexpressed in the basalis of pre-menopausal and post-menopausal endometrial epithelial cells, the putative residence of epithelial stem/progenitor cells. Given that N-cadherin and P-cadherin are also associated with the Wnt signalling gene pathway (Chapter 3), they were chosen as markers for investigation. Further, N-cadherin is a marker of the haematopoietic stem cell niche (Zhang *et al.*, 2003). Both N-cadherin and P-cadherin isolated a small endometrial epithelial cell population with cloning efficiencies of 0.6% and 0.4% respectively. Interestingly, N-cadherin^{pos} cells but not P-cadherin^{pos} cells exhibited the ability to self-renew, the essential hallmark of stem cells, indicating that N-cadherin^{pos} cells are possibly rare, quiescent stem cells with capacity for self-renewal and clonogenicity, while their transit-amplifying progeny could undergo rapid proliferation, which also produce CFU. In addition, N-cadherin expressing cells had a 1.8 fold increased in CFU compared to unfractionated epithelial clonogenic cells identified earlier (Chan *et al.*, 2004). N-cadherin^{pos} clones were

cytokeratin positive and had a small compact morphology resembling large clones (Gargett *et al.*, 2009), and cells with high nuclear: cytoplasmic ratio, another stem cell property.

N-cadherin localised to epithelial cells in post-menopausal glands and in the deep basalis layer of pre-menopausal endometrium, nearer to the myometrial junction and gradually decreased toward the functionalis in keeping with the gene array data and suggesting a basalis niche for self-renewing clonogenic epithelial cells. The prominent basalis location of N-cadherin in pre-menopausal and post-menopausal endometrium is in contrast with previous findings reporting weak or absent of N-cadherin expression (Poncelet *et al.*, 2002; Tsuchiya *et al.*, 2006; Poncelet *et al.*, 2010). This is because previous studies have examined endometrial tissue sections obtained from curettage or biopsy rather than full thickness endometrium containing the basalis layer, in which N-cadherin expression is primarily localised. One gene profiling study found N-cadherin up-regulation in human endometrial cultured cells treated with estrogen (Pole *et al.*, 2005), while another study found down-regulation of N-cadherin in early secretory compared to proliferative endometrium, suggesting N-cadherin is inhibited by progesterone (Talbi *et al.*, 2006). Together, these studies indicate that N-cadherin fluctuates during the menstrual cycle. N-cadherin expression in basalis of pre-menopausal and post-menopausal endometrium in this study indicates that N-cadherin may respond to hormones to modulate endometrial epithelial cell proliferation and differentiation. It is likely that N-cadherin is involved in endometrial regeneration and this hypothesis warrants further investigation.

In contrast, P-cadherin possibly selected the more mature transit-amplifying cells. P-cadherin expression was found in nearly all the gland profiles of full thickness pre-menopausal and post-menopausal endometrium. P-cadherin expressing cells did not enrich for endometrial epithelial cells with stem cell properties, limiting its potential as a marker

of endometrial epithelial stem/progenitor cells. However, P-cadherin localisation to all endometrial glands confirmed previous studies suggesting that P-cadherin is important in the maintenance of endometrial epithelial architecture and proliferation (van der Linden *et al.*, 1994; van der Linden *et al.*, 1995).

The identification of N-cadherin as a putative marker is the first step toward understanding endometrial epithelial stem/progenitor cell biology. While this study is still preliminary, it lays the foundation for further studies into the characterisation of human endometrial epithelial stem/progenitor cells. One immediate study of N-cadherin positive cells is to examine their ability to differentiate into three-dimensional gland-like structures. A previous study from our laboratory has demonstrated that clonally derived endometrial epithelial cells generated large cytokeratin positive expressing gland-like structures in 3D culture (Gargett *et al.*, 2009). Evidence has shown that the 3D cultures resembled the microenvironment similar to that of human body (Zietarska *et al.*, 2007; Härmä *et al.*, 2010) and that a 3D Matrigel culture system supports mammary epithelial stem/progenitor cells differentiation into functional, mature progeny (Lim *et al.*, 2009). Additionally, *in vivo* tissue reconstitution of transplanted N-cadherin positive cells should also be investigated. Unfractionated endometrial single cell suspensions reconstitute endometrial-like tissue when transplanted into super-immunocompromised NOG mice that responds to estrogen and progesterone hormones (Masuda *et al.*, 2007). Another approach to assess the validity of N-cadherin as a marker of endometrial epithelial stem/progenitor cells is to examine whether they are present in the side population cells, given that side population cells exhibited stem cell properties and have been identified in the endometrium (Kato *et al.*, 2007; Tsuji *et al.*, 2008; Cervello *et al.*, 2010; Masuda *et al.*, 2010; Cervello *et al.*, 2011). Demonstration of *in vitro* and *in vivo* differentiation of N-cadherin^{pos} cells and their presence in the side population would strengthen the CFU and *in vitro* self renewal

evidence provided in this thesis that the N-cadherin^{pos} cells are indeed endometrial epithelial stem/progenitor cells.

Accumulating published evidence (Chan *et al.*, 2004; Schwab *et al.*, 2005; Gargett *et al.*, 2009; Masuda *et al.*, 2010) and this study support the presence of endometrial epithelial stem/progenitor cells in the endometrium. However, descriptive morphological studies have challenged this concept and suggested that epithelial stem/progenitor cells are a mere product of endometrial stromal cell differentiation (Baggish *et al.*, 1967; Garry *et al.*, 2009). Given that N-cadherin as a candidate marker and that Wnt signalling is likely involved in endometrial epithelial stem cell as their niche, further investigation will allow the elucidation of the origin and location of endometrial epithelial stem/progenitor cells.

6.4 Candidate markers of endometrial cancer stem cells

Known cancer stem cell markers (CD24, CD29, CD49f and CD44) from other systems (Al-Hajj *et al.*, 2003; Dalerba *et al.*, 2007; Li *et al.*, 2007; Gao *et al.*, 2010) were expressed by both primary type I endometrial cancer cells and in several endometrial cancer cell lines, ECC1, Ishikawa and HEC-1A (Chapter 5). The level of expression was more similar between the endometrial cancer cell lines and different to primary endometrial cancer cells. All sorted subpopulations (EpCAM/CD29, EpCAM/CD49f and EpCAM/CD44) of EnCa cell lines, but not primary EnCa cells, formed colonies. We found that flow cytometry sorting had profound effects on cell survival of freshly isolated primary EnCa sorted cells. Consequently, it was not possible to compare and short list candidate markers for further study in the limited time available for this thesis.

Given that the endometrium and the breast are hormonally regulated (Jabbour *et al.*, 2006; Asselin-Labat *et al.*, 2010) and that breast cancer stem cells were isolated using

$CD44^{pos}CD24^{neg}$ (Al-Hajj *et al.*, 2003), the CD44/CD24 subpopulations were examined. To our surprise, $CD44^{pos}CD24^{neg}$ identified a small population of endometrial cancer cells with colony-forming activity which was enhanced when grown on stromal feeder layer support. This data suggests that $CD44^{pos}CD24^{neg}$ population potentially identifies the endometrial cancer stem cell sub-population. However, stem cell self-renewal property must be demonstrated to confirm this finding. In addition, $CD44^{pos}CD24^{neg}$ cells and other sub-populations should also be compared for their ability to initiate tumours *in vivo* to warrant their stem cell potential. Sphere formation was also demonstrated in this study, and together with CFU activity this study supports the existence of rare stem cells in endometrial cancer (Friel *et al.*, 2008; Hubbard *et al.*, 2009). Another conclusion drawn from this study is that endometrial cancer cell lines contain a homogenous population of cells expressing most candidate markers with similar CFU activity. This indicates that cell lines examined in this thesis were not suitable for identifying endometrial cancer stem cell markers.

Concurrent research has identified CD133 as a putative marker of endometrial cancer stem cells (Rutella *et al.*, 2009), however, these sorted cells were incapable of tumour formation *in vivo*. Further evidence from our laboratory indicated that CD133 does not enrich for endometrial cancer stem cells (Hubbard, 2010). In addition, an immunohistological study identified Musashi-1 as a potential marker (Götte *et al.*, 2008), however stem cell functional assays are required to confirm this claim.

Given that cancer stem cells could arise from normal stem cells with altered genetic and/or epigenetic profiles and that they exhibit similar properties as normal adult stem cells, albeit unregulated (Reya *et al.*, 2001; Pardal *et al.*, 2003; Jordan *et al.*, 2006), N-cadherin could be used to isolate endometrial cancer cells and examining their stem cell activity. The

advantage of specific markers for isolating cancer stem cells is evident in the mouse and mammary stem cells literature which has revealed distinct pathways involved in normal development and tumorigenesis (Lim *et al.*, 2010). Yet at the same time, there are conserved genes that are shared between normal and tumour subsets, indicating their contribution to tumourigenicity, and that normal and cancer stem cells utilise similar pathway to regulate stem cell fate decisions (Lim *et al.*, 2010). Consequently, N-cadherin is potentially an ideal marker that could be used to isolate epithelial stem/progenitor cells from normal endometrium and endometrial cancer to study and compare their molecular and cellular phenotypes and functions.

6.5 Limitations of the investigations

The main limitation of this study was the variation in the availability of endometrial tissue samples. The average number of samples varied from 2-3 per week to 0 samples for 4-5 weeks. This variation of sample availability interfered with data collection for this investigation. Additionally, these samples had to be shared with other scientists. The size and number of single cells that could be isolated from a sample, particularly post-menopausal samples also limited their use in undertaking flow cytometry sorting and stem cell functional assays.

Much time (one year) was dedicated to set up and optimise the best conditions for sorted endometrial epithelial cells, including optimisation for sorting buffer, antibody concentration, and culture medium and coating matrices because when this study begun, there were no flow cytometry protocols available to sort endometrial epithelial cells or endometrial cancer cells. This component of the thesis has, however, achieved the best conditions for flow cytometry sorting, a lasting legacy for those who will continue to work

on endometrial epithelial stem/progenitor cells. A further challenge was the poor cell survival and growth after sorting when seeded at clonal density. Subsequently, time was spent on optimising two alternative approaches to address this problem. This included i) the use of stromal conditioned medium and/or stromal feeder layers to support flow cytometry sorted cells and ii) magnetic bead sorting instead of flow cytometry sorting. Therefore, overcoming these difficulties contributes to the development of new knowledge in the emerging field of endometrial stem cell biology research. The knowledge generated in this study has demonstrated that endometrial epithelial stem/progenitor cells are likely locate in the basalis and could be tightly regulated by the Wnt signalling pathway within a stem cell niche. Alterations to this process could result in gain of mutations, resulting in the formation of cancer stem cells responsible for generating and progressing endometrial cancer. Data from this investigation also suggests that basalis epithelium of pre-menopausal women is similar to post-menopausal endometrial epithelium, evidenced by the sharing of a common gene signature.

6.6 Implications for gynaecological diseases

It is known that abnormal endometrial proliferation is a common feature of gynaecological diseases including endometriosis, endometrial hyperplasia, endometrial cancer and adenomyosis, yet their aetiology is poorly understood. It has been suggested that the aberrant alteration in endometrial stem/progenitor cells or their niche may be responsible for the initiation and progression of these diseases (Gargett, 2004, 2007).

The identification of the differentially expressed Wnt signalling genes and N-cadherin in this study provides an immediate foundation to apply this knowledge to endometrial cancer

and further characterise and confirm a role of cancer stem cells in this common reproductive tract cancer in women.

Endometriosis is a common gynaecological disorder affecting 1 in 10 reproductive-aged women (Rogers *et al.*, 2009). This estrogen-dependent disease likely involve retrograde menstruation to distribute shedding endometrial tissue outside the uterus into the pelvic cavity as originally theorised by Sampson (Sampson, 1927; Giudice & Kao, 2004). Recent emerging evidence suggests that endometrial stem/progenitor cells are also shed in menstrual blood (Meng *et al.*, 2007; Patel *et al.*, 2008) and that subsequent studies have confirmed this hypothesis with the identification of rare clonogenic cells in endometriotic lesions and peritoneal fluid (Chan *et al.*, 2011; Tan, 2011). The evidence provided in this thesis suggests that menstrual blood, peritoneal fluid and endometriosis lesions should be examined for putative N-cadherin positive endometrial epithelial stem/progenitor cells. A previous study found N-cadherin expression on epithelial cells isolated from an endometriosis biopsy (Zeitvogel *et al.*, 2001). Taken together, N-cadherin could be an invaluable tool in assessing the role of stem/progenitor cells in endometriosis and indeed work has commenced in our laboratory using the discovery from this thesis. This is important and warrants further investigation to elucidate the pathogenesis of endometriosis involving endometrial stem/progenitor cells.

Adenomyosis is defined by the invasion of endometrial glands and stroma deep into the myometrium (Ferenczy, 1998). It has been hypothesised to involve the alterations in the stem cell niche signalling to stem/progenitor cells, which results the migration of differentiated progeny into the myometrium (Gargett, 2007). The data generated from this investigation could be used to determine the role of endometrial epithelial stem/progenitor cells in adenomyosis.

6.7 Conclusions

The human endometrium has immense regenerative capacity but despite this fact it has been overlooked and under-recognised for many years as an ideal tissue for investigating adult stem cell activity. Work from this thesis, particularly the identification of a potential endometrial epithelial stem/progenitor cell marker will significantly contribute to our understanding of the cyclical processes of endometrial regeneration and regression. In particular, the prospective isolation of endometrial epithelial stem/progenitor cells will indeed extend our knowledge of their origin and location. This thesis provides evidence showing the common gene signature shared by basalis epithelium of pre-menopausal and post-menopausal endometrium which could regulate endometrial regeneration. The data generated from this thesis also supports the theory that endometrial epithelial stem/progenitor cells are located in the basalis and in postmenopausal endometrium, and that cancer stem cells may contribute to endometrial cancer.

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Appendices

Appendix 1 Other peer reviewed publications during PhD

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Isolation and Culture of Epithelial Progenitors and Mesenchymal Stem Cells from Human Endometrium[1]

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ABSTRACT

Human endometrium is a highly regenerative tissue undergoing more than 400 cycles of growth, differentiation, and shedding during a woman's reproductive years. Endometrial regeneration is likely mediated by adult stem/progenitor cells. This study investigated key stem cell properties of individual clonogenic epithelial and stromal cells obtained from human endometrium. Single-cell suspensions of endometrial epithelial or stromal cells were obtained from hysterectomy tissues from 15 women experiencing normal menstrual cycles, and were cultured at clonal density (10 cells/cm^2) or limiting dilution. The adult stem cell properties—self-renewal, high proliferative potential, and differentiation of single epithelial and stromal cells—were assessed by harvesting individual colonies and undertaking serial clonal culture, serial passaging, and culture in differentiation-induction media, respectively. Lineage differentiation markers were examined by RT-PCR, immunocytochemistry, and flow cytometry. Rare single human endometrial EpCAM$^+$ epithelial cells and EpCAM$^-$ stromal cells demonstrated self-renewal by serially cloning >3 times and underwent >30 population doublings over 4 mo in culture. Clonally derived epithelial cells differentiated into cytokeratin$^+$ gland-like structures in three dimensional culture. Single stromal cells were multipotent, as their progeny differentiated into smooth muscle cells, adipocytes, chondrocytes, and osteoblasts. Stromal clones expressed mesenchymal stem cell (MSC) markers ITGB1 (CD29), CD44, NT5E (CD73), THY1 (CD90), ENG (CD105), PDGFRB (CD140B), MCAM (CD146) but not endothelial or hemopoietic markers PECAM1 (CD31), CD34, PTPRC (CD45). Adult human endometrium contains rare epithelial progenitors and MSCs, likely responsible for its immense regenerative capacity, which may also have critical roles in the development of endometriosis and endometrial cancer. Human endometrium may provide a readily available source of MSCs for cell-based therapies.

adult stem cells, clonal assays, differentiation, endometrial stem cell, epithelial progenitor cell, female reproductive tract, human endometrium, mesenchymal stem cell, uterus

INTRODUCTION

The human endometrium is a dynamic remodeling tissue undergoing more than 400 cycles of regeneration, differentiation, and shedding during a woman's reproductive years [1–3]. Each month, 4–7 mm of mucosal tissue grows within 4–10 days in the first half or proliferative stage of the menstrual cycle [3]. Endometrial regeneration also follows parturition, extensive resection, and occurs in postmenopausal women taking estrogen replacement therapy [1]. This level of new tissue growth is at least equivalent to the cellular turnover in other highly regenerative organs, such as blood-forming tissue of the bone marrow, epidermis, and intestinal epithelium, where adult stem cells replenish lost cells to maintain tissue homeostasis [4, 5].

Adult stem cells are rare, undifferentiated cells present in adult tissues and organs. They are extremely difficult to identify in tissues, as they are rare, lack distinguishing morphological features, and specific adult stem cell markers are currently unavailable. Adult stem cells are therefore defined by their functional properties: substantial self-renewal, high proliferative potential, and ability to differentiate into one or more lineages [6, 7]. These functions are highly regulated by the stem cell niche to ensure an appropriate balance between stem cell replacement and provision of sufficient differentiated mature cells for tissue and organ function [7, 8].

There is increasing interest in the concept that endometrial stem/progenitor cells may be responsible for the highly regenerative capacity of human endometrium. It has been hypothesized that both epithelial and stromal adult stem cells exist in the basal layer of human endometrium, since regeneration occurs from this layer after the top two-thirds or functional layer is shed at menstruation, and the endometrium comprises glandular tissue supported by an extensive vascularized stroma [1, 9]. Initial evidence from cell cloning studies suggests that adult stem cells are likely present in human endometrium [10, 11], but subsequent studies have focused on various subpopulations of epithelial and/or stromal cells rather than individual cells [12–15]. The pluripotency marker, POU5F1 (formerly Oct-4) has been observed in some cells in human endometrial stroma [16], but the identity and stem cell function of these cells was not examined. To date, adult stem cell activity of individual human endometrial epithelial and stromal cells has not been investigated.

Disorders of uterine endometrial proliferation are common, leading to endometriosis, endometrial hyperplasia, and endometrial cancer. Despite their common occurrence and the substantial public health burden that these diseases present [17], little is known about their pathogenesis [18–20]. We

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hypothesize that endometrial stem or progenitor cells play key roles in the initiation of these endometrial proliferative disorders [1]. In endometriosis, endometrial stem/progenitor cells may be shed into the pelvic cavity by retrograde menstruation to establish endometriotic growths [1]. Endometrial epithelial progenitors or their immediate progeny may be targets of early genetic or epigenetic alterations, leading to the emergence of endometrial cancer stem cells that initiate and maintain endometrial cancer [1, 19]. In this study, we report the isolation of individual epithelial progenitor cells and mesenchymal stem cells (MSCs) in human endometrium and the characterization of their adult stem cell properties of self-renewal, high proliferative potential, and differentiation.

MATERIALS AND METHODS

Human Tissues

Human endometrium was obtained from hysterectomy samples collected from 15 cycling women, aged 31–49 yr (mean, 41.3 ± 1.3 [±SEM]) undergoing surgery for fibroids and/or adenomyosis and prolapse (Table 1), and who had not taken exogenous hormones for 3 mo prior to surgery. Endometrial tissue was collected distal to submucosal fibroids if they were present. The study protocol was approved by the Southern Health Human Research and Ethics Committee B, and informed written consent was obtained from each patient. The women reported the date of their last menstrual period, and menstrual cycle stage was confirmed from pathology reports assessed by experienced histopathologists, according to well-established histological criteria for the normal menstrual cycle [21]. Patient details, including menstrual cycle stage at time of hysterectomy, are listed in Table 1. Full thickness endometrium attached to 5 mm myometrium was collected in medium containing Hepes-buffered Dulbecco modified Eagle medium (DMEM)/Hams F-12 (F-12) (Invitrogen, Carlsbad, CA) containing antibiotics and 5% newborn calf serum (CSL, Parkville, Australia) [10], stored at 4°C, and processed within 2–18 h.

Human endometrial tissue was scraped from the myometrium and dissociated into single-cell suspensions using collagenase type 3 (300 μg/ml; Worthington Biochemical Corp., Freehold, NJ), 40 μg/ml deoxyribonuclease type I (Roche Diagnostics, Mannheim, Germany), and mechanical methods for 50–60 min [10]. Leukocytes were removed with anti-PTPRC (CD45)-coated Dynabeads (Dynal Biotech, Oslo, Norway). Purified epithelial and stromal cell suspensions were then obtained by selecting epithelial cells with a further round of magnetic bead sorting using anti-EpCAM-coated Dynabeads [10]. Both epithelial and stromal cell preparations were >95% pure.

Clonal Cell Culture, Self-Renewal, and Proliferative Potential Assays

Purified, freshly isolated epithelial and stromal cells were cultured separately at clonal density (8–20 cells/cm^2, 500-1200 cells/dish) in 12 ml DMEM/F-12 medium containing 10% fetal calf serum (FCS; CSL), 2 mM glutamine (Invitrogen), and antibiotic-antimycotic on fibronectin-coated 100-mm petri dishes (10 μg/ml; Becton Dickinson Biosciences, Bedford, MA) in triplicate, and in limiting dilution or at 1 cell/well in 96-well plates (100 μl medium/well using a stock cell suspension of 10 cells/ml) and incubated at 37°C in 5% CO_2. The culture medium for epithelial cells was also supplemented with 10 ng/ml epidermal growth factor (GroPep, Adelaide, Australia) to promote growth of epithelial clones [10]. A seeding density of 8–20/cm^2 was chosen based on our previous cloning efficiency data [10] to ensure that no more than 10 well-separated, nonoverlapping clones/plate were obtained. Plates were examined twice/week to ensure clones were established from single cells, and individual colonies were monitored until harvest. For the limiting dilution analysis, freshly isolated epithelial and stromal cells were seeded in 100-μl volumes into wells of 96-well plates with 8 replicates/cell concentration for 4 patient samples in serial dilution from 256 to 0.5 cells/well from stock cell suspensions of 2560–5 cells/ml. Due to the low number of cells in culture plates, medium changes were done every 14 days, except for the higher dilutions in the 96-well plates, where weekly or more frequent changes were required. Following fixation in 10% formalin and staining with 0.5% toluidine blue, colony efficiency assays were performed using Poisson distribution statistics by determining the percentage of wells without cell clones (>50 cells) after 30 days in culture using limiting dilution software tools in the statmod software package for R computing environment (available at http://cran.r-project.org/). The R version (R2.7.0) of the limdil software (http://bioinf.wehi.edu.au/software/elda/index.html) was used for analysis.

TABLE 1. Details regarding patients in this study.

Sample no.	Age (yr)	Menstrual cycle stage	Reason for hysterectomy
1	41	Early secretory	Prolapse
2	45	Proliferative	Leiomyomata
3	42	Secretory	Leiomyomata
4	45	Proliferative	Leiomyomata
5	34	Not reported	Leiomyomata
6	31	Early secretory	Adenomyosis
7	49	Interval	Adenomyosis
8	47	Proliferative	Leiomyoma
9	42	Proliferative	Leiomyomata
10	43	Proliferative	Unknown
11	39	Proliferative	Leiomyoma
12	44	Proliferative	Prolapse
13	44	Late secretory	Adenomyosis, leiomyoma
14	38	Early secretory	Leiomyomata
15	35	Proliferative	Prolapse

Nonoverlapping single epithelial or stromal clones were harvested from culture dishes using 0.025% trypsin (Invitrogen) and cloning rings (Sigma-Aldrich, St. Louis, MO) after 20–35 days in culture, or from individual wells containing a single colony (Fig. 1, A and B). Six to twelve large clones containing 4–8 × 10^3 epithelial or stromal cells, or 6–12 small clones comprising 0.5–2 × 10^3 cells, were collected from each patient sample for analysis of adult stem cell properties. A small proportion of the cells in the harvested clones were cultured on coverslips for cytokeratin or α_6 integrin (IGTA6), and THY1 (CD90) immunocytochemistry analysis (Table 2) [10] to confirm that individual clones were epithelial or stromal, respectively, prior to undertaking adult stem cell assays.

Self-renewal of epithelial and stromal cells was assessed by serial cloning individual large and small clones generated by single epithelial or stromal cells. Cells from individual clones were reseeded at cloning density (5–10 cells/cm^2) in 100-mm petri dishes to generate secondary clones. Two stromal and two epithelial clones were harvested per primary clone 14 and 21 days later,

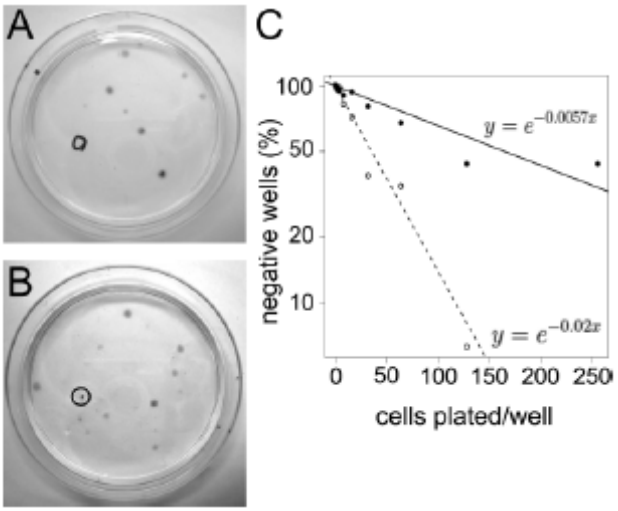

FIG. 1. Human endometrial cell clones. Typical 6-cm cloning plates of freshly isolated single cell suspensions of epithelial (**A**) and stromal (**B**) cells seeded at 20 and 10 cells/cm^2, respectively, showing well-separated individual colonies (CFU). Circles indicate removal of a clone for analysis. **C**) Limiting dilution analysis showing frequency of epithelial (black circles) and stromal (open circles) clones in endometrial cell suspensions by limiting dilution. Data are from four patient samples (nos. 11, 12, 14, and 15), with eight replicates/sample using Poisson distribution analysis. The frequency of stromal CFU was significantly greater than for epithelial ($P < 0.0001$).

TABLE 2. Antibodies used to phenotype human endometrial cells by flow cytometry and immunocytochemistry.

Primary antibodies	Isotype	Clone/fluorochrome	Concentration	Source
CD29 (ITGB1)	Rat IgG_{2a}	mAb 13	1 μg/ml	Becton Dickinson, Bedford, MA
CD31 (PECAM)	Mouse IgG_1	JC/70A	4 μg/ml	Dako, Glostrup, Denmark
CD34	Mouse IgG_1	581/PE-Cy5.5	50 μl/ml	Southern Biotech, Birmingham, AL
CD44	Mouse IgG_{2b}	G44–26	1 μg/ml	Becton Dickinson, Bedford, MA
CD45 (PTPRC)	Mouse IgG_1	HI30/APC	10 μg/ml	Caltag, Burlingame, CA
CD73 (NT5E)	Mouse IgG_1	AD2	20 μg/ml	Becton Dickinson, Bedford, MA
CD90 (THY1)	Mouse IgG_1	5E10/FITC	1 μg/ml (FC)* 4 μg/ml (ICC)†	Becton Dickinson, Bedford, MA
CD105 (ENG)	Mouse IgG_1	266	10 μg/ml	Becton Dickinson, Bedford, MA
CD146 (MCAM)	Mouse IgG_{2a}	CC9	500 μl/ml	Gift from Australian Stem Cell Centre, Melbourne, Australia
STRO-1	Mouse IgM	STRO-1	1 μg/ml	R&D Systems, Minneapolis, MN
Cytokeratin	Mouse IgG_1	MNF116	2 μg/ml	Dako, Glostrup, Denmark
ITGA6 (α_6 integrin)	Rat IgG_{2A}	GoH3	10 μg/ml	Becton Dickenson, Bedford, MA
ACTA2 (αSMA)	Mouse IgG_{2a}	1A4	3.6 μg/ml	Dako, Glostrup, Denmark

* FC, flow cytometry.
† ICC, immunocytochemistry.

respectively, since clonogenic stromal cells proliferated more rapidly than clonogenic epithelial cells. Each clone was subcloned into duplicate 100 mm culture plates if >600 cells were harvested from the primary clone, or subcloned into a single dish for secondary or higher-order clones containing <500 cells. The secondary clones were reclonal in a similar manner to generate tertiary clones (Fig. 2), and reclonal of individual clones continued until cloning activity was exhausted. Some cells from each clone were plated on coverslips, and their epithelial and stromal phenotype confirmed by immunocytochemistry for cytokeratin and THY1 (CD90) (Table 2), respectively.

Proliferative potential of individual colony-forming epithelial and stromal cells was assessed by serially passaging [22, 23] 3–5 large and 3–5 small primary clones per patient sample at bulk culture seeding density (2000 cells/cm^2) until senescence. Progressively larger wells and flasks were used with each passage, as each clone formed an individual cell line. Cells were passaged every 7–10 days when cultures reached 80–100% confluence. Once sufficient cells were obtained for each cell line, duplicate cultures were established for each clone and excessive cells were frozen in 10% DMSO/90% FCS and stored in liquid nitrogen. Cell counts were done at each passage, and cumulative population doublings (cPDs) calculated from the formula: cPD = ln(cumulative cell yield)/ln2 [22]. At each passage, a small proportion of cells was seeded at clonal density (25–50 cells/cm^2) in 60-mm plates, and the cloning efficiency at each passage was calculated: (no. clones/no. cells seeded) × 100 [22].

In Vitro Differentiation

Clonally derived epithelial cells. Large primary or secondary epithelial clones were expanded in culture and 2×10^5 cells were then cultured in 1:1 dilution of Matrigel (Becton Dickinson) in DMEM/F12/10% FCS medium in eight-well chamber slides [24] placed over a monolayer of endometrial stromal cells cultured in the same medium in 5% CO_2 at 37°C for 25 days. Culture medium was changed twice weekly. Differentiation into gland-like structures was monitored microscopically, and cultures were immunostained with anti-human cytokeratin (2 μg/ml; Dako, Carpinteria, CA). An isotype-matched IgG negative control (Table 2) was included for each sample examined. The area of gland-like structures was measured using Zeiss AxioVision 4.6 image analysis software.

Clonally derived stromal cells. Large primary or secondary stromal clones were isolated, expanded in culture in serum-containing medium (DMEM/F12/10% FCS) to generate $2–3 \times 10^6$ cells, seeded at 5000–10 000 cells/cm^2 into 25-cm^2 flasks and 13-mm gelatin-coated Thermanox coverslips (Nalge Nunc, Naperville, IL), and cultured in specific differentiation-induction media (Table 3) for 4 wk using standard methods [25, 26]. Media were changed every 2–3 days. Undifferentiated control cells were cultured concurrently in low serum medium (DMEM/F12/1% FCS%/1% antibibiotic/1% glutamine) for the same incubation time. Control media were changed as regularly as the differentiation induction media. Total RNA was isolated from cultures to assess cell lineage-specific genes by RT-PCR, and cells on coverslips were stained by histochemical or immunocytochemical methods [12]. Isotype-matched IgG-negative controls were included for each antibody (Table 2). Osteogenic differentiation was assessed using an alkaline phosphatase kit (Sigma-Aldrich) and parathyroid hormone receptor 1 (*PTHR1*) mRNA using primers listed in Table 3 [12]. Adipogenic differentiation into lipid-laden cells was detected with Oil Red O and the adipogenic lineage-specific gene, lipoprotein lipase (*LPL*) (Table 3) [12]. Myogenic differentiation was induced by culturing cells in medium supplemented with heat-inactivated 5% male human serum (Red Cross Blood Service, Melbourne, Victoria, Australia) and 50 μM hydrocortisone (Sigma-Aldrich). Smooth muscle cells were detected by smooth muscle actin (αSMA; 3.6 μg/ml, clone 1A4; Dako) immunocytochemistry and expression of caldesmon mRNA (Table 3) [12]. Chondrogenic differentiation was detected in 5-μm paraffin sections of formalin-fixed cell pellets incubated in 1% acidified Alcian blue, and by expression of collagen type II mRNA (Table 3) [12].

RT-PCR Analysis

Total RNA was isolated using Trizol (Invitrogen) with genomic DNA removed. RNA quality was assessed by spectrophotometry, and the nucleotide:protein ratio (260:280) was within acceptable boundaries of 1.8 and 2.1. A 1-μg aliquot was reverse transcribed into cDNA with AMV reverse transcriptase (Roche, Penzberg, Germany) at 42°C for 1 h. Complementary DNA was amplified using GoTaq Green Master Mix (Promega) in a Gene Amp PCR System 2700 (Applied Biosystems, Foster City, CA). Primer sequences are shown in Table 3; 18S RNA was the loading control. Reaction products were analyzed by 1.5% agarose gel electrophoresis and ethidium bromide. The sequence of each product was confirmed by automatic sequencing.

Flow Cytometry

Cells derived from secondary stromal clones were incubated with directly conjugated or unconjugated antibodies to MSC surface markers (Table 2) or matched-isotype control IgG for 45 min at 4°C, followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgM (10 μg/ml; Southern Biotech, Birmingham, AL), Alexa Fluor 488-conjugated chicken anti-rat IgG (10 μg/ml; Molecular Probes, Eugene, OR), or PE-conjugated sheep anti-mouse Ig $F(ab')_2$ fragments (10 μl/ml; Chemicon Australia, Melbourne, Victoria, Australia). Cells were incubated with propidium iodide (Sigma-Aldrich) and analyzed by flow cytometry using a Mo-Flo Cytometer (Cytomation, Fort Collins, CO) [12].

Statistical Analysis

Data are shown as mean ± SEM from n patient samples. Unpaired *t*-tests were used to compare the significance between two groups. Data for cell yield were log transformed prior to *t*-test analysis. Results were considered statistically significant at a P value <0.05.

RESULTS

In Vitro Self-Renewal of Endometrial Colony Forming Units/Cells

To assess the adult stem cell activity of individual endometrial cells, single-cell suspensions of freshly isolated endometrium (n = 15) were separated into $EpCAM^+$ epithelial cells and $EpCAM^-$ stromal cells and seeded at much lower cloning densities (10–20 cells/cm^2; Fig. 1, A and B) than in our previous studies [10], or at <1 cell/well in 96-well plates (n =

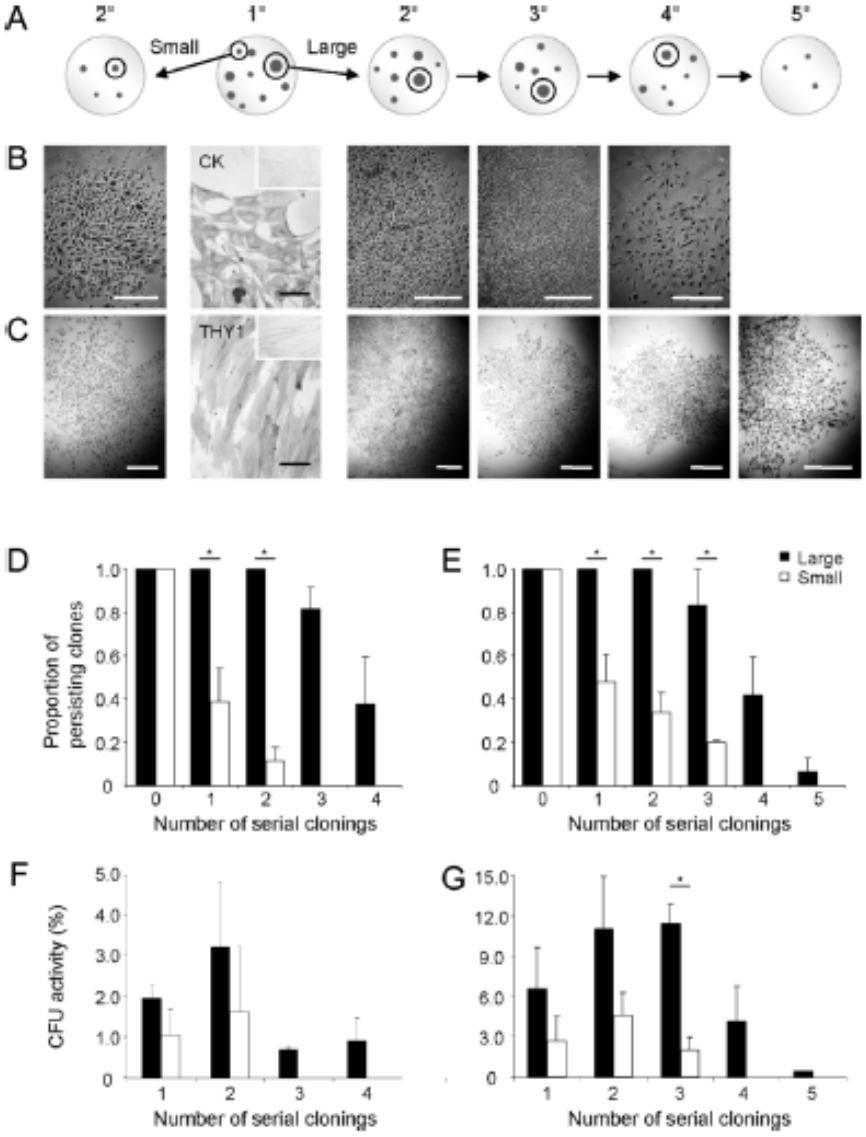

FIG. 2. Serial cloning analysis for measuring self-renewal of human endometrial large and small primary (1°) epithelial and stromal CFU. **A**) Schematic showing serial cloning strategy. The initial cloning plate (seeded at 10–20 cells/cm^2), second panel with two cloning rings selecting the largest (>4000 cells), and a small to medium (<2000 cells) CFU. These clones were individually replated at 5–10 cells/cm^2 and cultured for 14 days. Serial clonal passaging (2°–5°) is depicted as cloning plates containing representative clones, with selection of typical large clones for the subsequent round of cloning indicated within cloning rings, until CFU activity was exhausted (4°/5°). Typical endometrial epithelial (**B**) and stromal (**C**) colonies formed at each round of serial cloning. Endometrial epithelial colonies were cytokeratin$^+$ (CK) and stromal colonies THY1 (CD90$^+$) (second panels). Inserts are isotype controls. Rate of clonal extinction is shown for both large and small CFU for each round of serial cloning for epithelial (**D**) and stromal (**E**) CFU. Percentage of CFU in large and small epithelial (**F**) and stromal (**G**) CFU at each round of serial cloning. Results are means ± SEM (n = 3 patient samples [from nos. 7–9 for epithelial and nos. 1–4 for stromal]; averages of three to five small and large CFU/cell type/patient sample). *Significant difference between large and small CFU ($P < 0.05$). Bars = 1 mm for clones (**C**); bars = 50 μm for immunostained images, including insets (**B** and **C**).

3) to obtain epithelial and stromal cell colony forming cells (CFU). We also conducted limiting dilution assays in 96-well plates on 4 patient samples. Poisson distribution analysis demonstrated that the frequency of clonogenic epithelial cells was 1/174 (confidence interval [CI] 1/235, 1/130) and 1/48 (CI 1/61, 1/39) for stromal cells (Fig. 1C), in broad agreement with cloning efficiencies obtained on culture dishes [10].

Self-renewal was assessed using a serial cloning strategy, since single CFU undergoing self-renewing cell divisions during colony establishment will form new clones on recloning [27]. We harvested and recloned at least 5 individual large and small clones per patient sample at 5–10 cells/cm^2 (Fig. 2, A–C), and demonstrated that large endometrial epithelial CFU exhibited significantly greater self-renewal capacity than small CFU, undergoing 2.9 ± 0.5 versus 0.5 ± 0.3 ($P = 0.0048$; n = 3) rounds of serial cloning. Similarly, large stromal CFU underwent 3.3 ± 0.4 rounds of cloning compared with small CFU (0.9 ± 0.2; $P = 0.0054$; n = 4). All large epithelial and stromal CFU were able to serially clone ≥3 rounds (Fig. 2, D and E), significantly more than small CFU, where 39% of epithelial clones and 47% of stromal clones were able to initiate CFU on the second round, while 36% of large epithelial and 41% of large stromal CFU initiated clones on the third round, and 7% of large stromal CFU did so on the fourth (Fig. 2, D and E). Large epithelial and stromal CFU formed densely packed clones for the first three rounds of serial cloning (Fig. 2, B and C, central panels), but initiated smaller, less dense clones on further recloning, which appeared similar to primary small clones (Fig. 2, B and C, far right panels). The proportion of persisting clones [28] from the original large CFU was significantly greater for both cell types compared with the small CFU (Fig. 2, D and E). There was a trend for increasing CFU activity in epithelial secondary clones and also for stromal clones for secondary and tertiary clones (Fig. 2, F and G), but this increase was mainly small compared with large clones, indicating that the large CFU produce more differentiated progeny with decreasing proliferative potential at each serial cloning step.

The number of PDs undergone by individual large epithelial or stromal CFU during 3–4 subclonings was 35–45 (data not

TABLE 3. Mesenchymal differentiation induction and detection of lineage specific markers.

Lineage	Differentiation-induction media components	Staining	mRNA expression by RT-PCR: Gene name (gene symbol)	GenBank accession no.	Primer sequences for PCR amplification*
Osteogenic	1α-25-dihydroxyvitamin-D3 (0.01 μM) Ascorbic acid-2-phosphate (50 μM) β-glycerophosphate (10 mM)	Alkaline phosphatase kit	Parathyroid hormone receptor 1 (*PTHR1*)	M64110	S: 5′-CCTCACCGTAGCTGTGCTCATCCT-3′ A: 5′-GCCCCTCCACCAGAATCCAGTAG-3′
Adipogenic	Isobutyl-methylxanthine (500 μM) Dexamethasone (1 μM) Insulin (10 μM) Indomethacin (200 μM)	Oil Red O (1%)	Lipoprotein lipase (*LPL*)	NM_000316	S: 5′-CAAAACTTGTGGCCGCCCTGTA-3′ A: 5′-GGGGACCCTCTGGTGAATGTGTGT-3′
Myogenic	Hydrocortisone (50 μM) Heat-inactivated male human serum (5%)	αSMA	Caldesmon	NM_000237	S: 5′-ACAGTCACCAAGTCCTACCAGAAGAATG-3′ A: 5′-CCTCCAGGGCGGCTGAAAGT-3′
Chondrogenic	Insulin (6.25 μg/ml) Ascorbic acid-2-phosphate (50 μM) Transforming growth factor-β1 (10 ng/ml)†	Acidified alcian blue (1%)	Collagen type II	NM_033150	S: 5′-CACTCCTGGCACTGATGGTCCC-3′ A: 5′-CTTCTCCCTTCTCGCCGTTAGCAC-3′

* S, sense; A, antisense.
† Transforming growth factor-β1 was supplied by Peptrotech Inc., Rocky Hill, NJ.

shown). A total of 12–13 PDs of the original CFU produced a primary clone between 4×10^3 and 8×10^3 cells, and the size of subsequent subclones ranged between 0.5×10^3 and 1×10^3 cells, representing a further 9–10 PDs for the second, third, and fourth subclonings. This number of PDs for individual large CFU cultured at clonal density is similar to that obtained from single large CFU cultured to senescence at bulk culture densities (see below, Table 4).

Proliferative Potential of Endometrial CFU

Proliferative potential was determined by serially passaging three to five individual large and small human epithelial and stromal CFU from three different patient samples until senescence to determine the total cell output of single endometrial CFU. Large CFU could be cultured for more than 4 mo, and produced significantly more progeny than small CFU (Fig. 3). The yield from single large epithelial CFU ranged from 8.3×10^9 to 9.2×10^{11} cells, while large stromal CFU produced between 5.5×10^6 and 6.1×10^{12} cells before reaching senescence (Table 4), significantly more than the respective small epithelial and stromal CFU. Some small CFU survived in culture for 1–2 mo, but many could not be passaged. Large CFU generated six to seven orders of magnitude more cells than small CFU for both cell types (Table 4). The number of PDs—one indicator of the proliferative potential of individual CFU—was significantly greater for large epithelial (34.5 ± 2.8; $n = 3$) and large stromal (30.4 ± 2.3; $n = 3$) CFU than the respective small CFU ($P < 0.005$ and $P < 0.001$, respectively) (Table 4). When small colonies reached senescence, large CFU had already undergone twice the number of PDs compared with small CFU (Fig. 3), indicating that the generation time was half that for small CFU. Since CFU maintain the proliferative capacity of cell cultures, we assessed their number at each passage to determine the number of cell generations [22] and demonstrated that large CFU underwent >3- to 4-fold more cell generations than did small CFU (Table 4).

Differentiation of Endometrial CFU

To examine the differentiation potential of single endometrial CFU, we harvested individual secondary epithelial and stromal clones (Fig. 2) and expanded them in culture to produce sufficient cells for differentiation induction. Small CFU were not examined as insufficient numbers of cells were produced (Table 4). Clonally derived epithelial cells formed small spheroid cytokeratin$^+$ structures in three-dimensional Matrigel cultures (data not shown), but when a stromal feeder layer was included below the Matrigel layer (Fig. 4, A and B), much larger cytokeratin$^+$ (Fig. 4, B and C) gland-like structures developed, indicating the importance of niche cells. These structures were 15× larger than similarly cultured fresh endometrial cells, and 12× larger than without the endometrial

TABLE 4. Total cell output from individual human endometrial large and small CFU.

Cell type	CFU type	Cell yield	PD	Cell generations	N*
Epithelial	Large	$5.9 \pm 2.9 \times 10^{11}$†	34.5 ± 2.8†	40.3 ± 3.1†	3
	Small	$4.5 \pm 3.5 \times 10^5$	12.1 ± 0.4	12.2 ± 0.8	3
Stromal	Large	$6.4 \pm 4.6 \times 10^{11}$†	30.4 ± 2.3‡	42.7 ± 6.6†	3
	Small	$4.5 \pm 2.2 \times 10^4$	12.5 ± 0.9	10.2 ± 1.9	4

* Number of patient samples from which 3–5 large and small individual epithelial and stromal CFU were serially passaged at 2000 cells/cm^2 until senescence.
† Difference between large and small CFU; $P < 0.005$.
‡ Difference between large and small CFU; $P < 0.001$.

stromal feeder layer, suggesting that single endometrial epithelial CFU have the capacity to differentiate into mature "glands" in vitro.

Large secondary stromal clones, originating from a single stromal CFU, demonstrated multipotency as their progeny differentiated into four mesenchymal lineages when cultured in typical differentiation-induction media. In myogenic medium, clonally derived stromal cells differentiated into αSMA- and caldesmon-expressing cells (Fig. 4D), suggesting smooth muscle rather than myofibroblast differentiation. In osteogenic medium, clonally derived stromal cells strongly expressed alkaline phosphatase and *PTHR1*, an osteogenic lineage-specific marker, indicative of osteogenic differentiation (Fig. 4E). Most clonally derived stromal cells cultured in adipogenic differentiation media accumulated lipid droplets, as observed by phase contrast microscopy and confirmed with Oil Red O staining. They also expressed the adipocyte gene, *LPL* (Fig. 4F). Clonally derived stromal cells cultured as a pelleted micromass for chondrogenic differentiation showed strong Alcian blue staining of cartilaginous matrix in paraffin-embedded sections (Fig. 4G), and expressed the chondrocyte-specific marker, collagen type II. Control cultures failed to form sufficient extracellular cartilaginous matrix to produce pellets. Similarly, mesenchymal lineage-specific markers were not observed in any control cultures (Fig. 4, D–G). These data suggest that single stromal CFU have MSC properties.

Phenotype of Clonally Derived Large Stromal CFU

Large secondary stromal CFU were then examined for typical MSC phenotypic surface markers. Flow cytometric analysis showed that the majority of clonally derived human endometrial stromal cells expressed high levels of the surface markers, ITGB1 (CD29), CD44, NT5E (CD73), and THY1 (CD90); approximately 20% expressed ENG (CD105) and MCAM (CD146), and were negative for PECAM1 (CD31), CD34, and PTPRC (CD45) (Fig. 5), indicating that they have a similar phenotype to bone marrow and fat MSCs, although they lacked Stro-1 expression (Fig. 5).

DISCUSSION

The human endometrium is a cyclically regenerating mucosal tissue comprising glands and an extensive vascularized stroma. Our findings show, for the first time, that rare individual endometrial cells with colony forming activity (large CFU) display adult stem cell properties of self-renewal, differentiation, and high proliferative potential in vitro. This suggests that they are responsible for monthly endometrial tissue regeneration, preparing the endometrium for steroid hormone-initiated differentiation into a receptive environment for embryo implantation. Both epithelial progenitor cell and MSC-like populations were identified. The entire endometrial functionalis layer, which is shed each month during menstruation, is likely replenished from these endometrial epithelial and stromal CFU, postulated to reside in the basalis. The small CFU, a more numerous population, have less proliferative potential, and are likely transit-amplifying cells, defined as proliferative stem cell progeny fated for differentiation, that also contribute to the rapid monthly growth of the endometrial mucosa.

Emerging evidence suggests that human and mouse endometrium may contain adult stem cells. Reports have identified clonogenic cells [10], side population (SP) cells [13, 29], and some cells that can differentiate into chondrocytes [15] or adipocytes [30] in human endometrium, while mouse endometrium contains label-retaining cells (LRC) [31, 32].

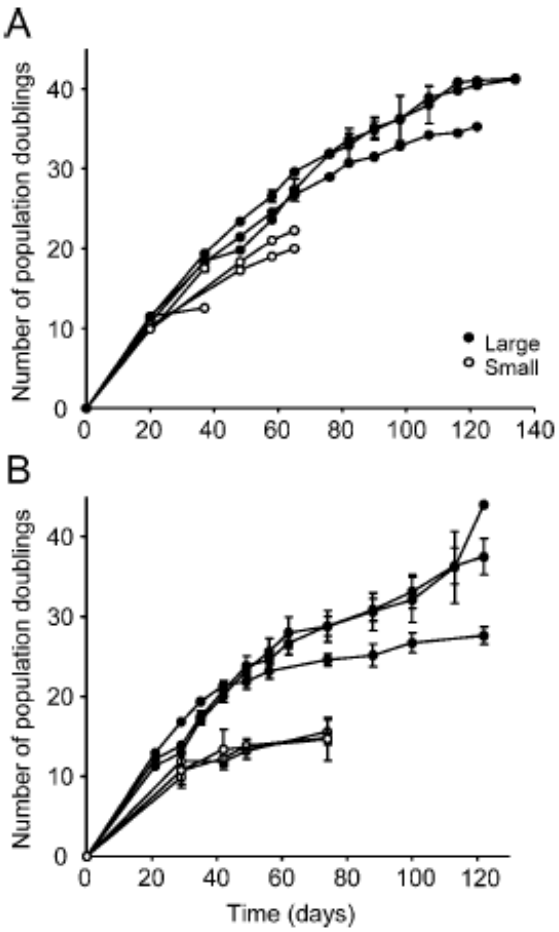

FIG. 3. Proliferative potential of human endometrial large and small epithelial and stromal CFU. Growth curves from single primary large and small epithelial (**A**) and stromal (**B**) CFU from individual patient samples passaged at 2000 cells/cm^2 illustrating differences in cell proliferation rates and total cell output. Each curve is the mean ± SEM of n = 3–5 clones derived from an individual patient sample (n = 3 patients, nos. 10, 12, and 13 for epithelial; n = 4 patients, nos. 1–4 for stromal).

However, identification of CFU, SP cells, or LRC alone in adult tissues is not sufficient to prove the existence of adult stem cells [33]. It is necessary to demonstrate key adult stem cell properties of individual putative stem cells. In the present study, we show that single, freshly isolated human endometrial epithelial cells initiating large epithelial CFU undergo substantial self-renewal in serial cloning or replating assays, have high proliferative potential, producing billions of cells, and undergo unilineage differentiation into gland-like organoids in vitro. These properties suggest that endometrial progenitor cells initiate the rare, large epithelial CFU of human endometrium. We also found that rare, single, freshly isolated endometrial stromal cells self-renew, have high proliferative potential, and undergo multilineage differentiation into four mesenchymal lineages in vitro, suggesting that they are similar to bone marrow MSCs [34]. Large endometrial stromal CFU undergo substantial self-renewal, producing tertiary or higher order clones, indicating that they are mesenchymal stem/progenitor cells [35]. Small epithelial and stromal CFU may be initiated by more differentiated transit-amplifying cell populations that progressively acquire differentiation markers as they

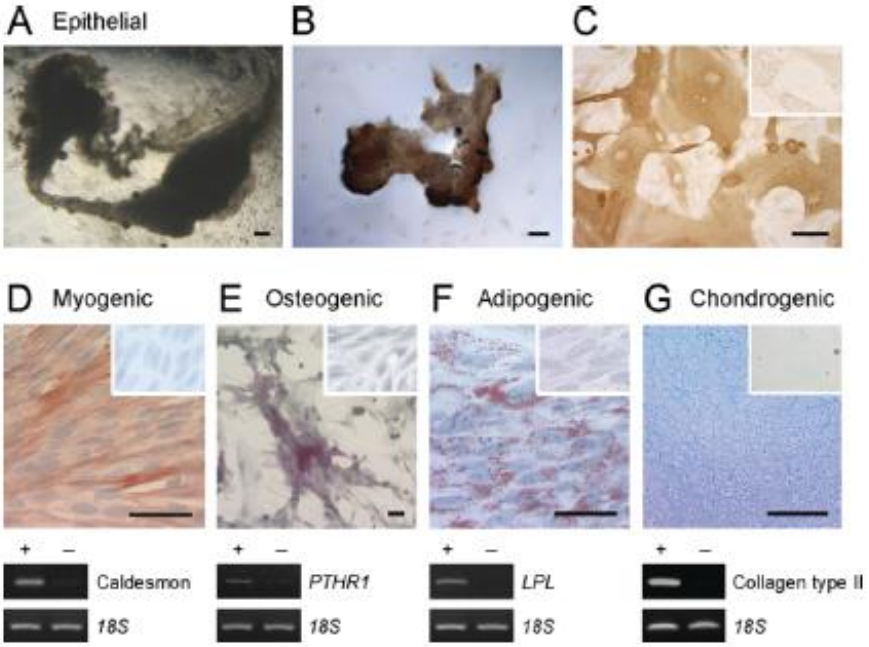

FIG. 4. Multilineage differentiation of single-cell-derived large human endometrial epithelial CFU and stromal CFU. **A–C**) Clonally derived epithelial cells (large CFU) cultured in 50% Matrigel above an endometrial stromal cell feeder layer for 4 wk differentiated into epithelial gland-like structures (**A**) observed by phase contrast microscopy, and (**B** and **C**) immunoreactive for cytokeratin. Inset in **C** is the isotype control. **D–G**) Clonally derived stromal cells (two clones) were cultured as monolayers or cell pellets (chondrogenic) for 4 wk in differentiation-induction media for mesenchymal lineages and assessed for lineage-specific markers by histochemistry, immunocytochemistry (upper panels), and RT-PCR (lower panels). **D**) Myogenic differentiation to smooth muscle cells, positive for αSMA and caldesmon. **E**) Osteogenic differentiation indicated by alkaline phosphatase reactivity and expression of parathyroid hormone receptor 1 (*PTHR1*). **F**) Adipogenic differentiation visualized by Oil Red O staining of lipid droplets and expression of lipoprotein lipase (*LPL*). **G**) Chondrogenic differentiation shown in a paraffin section of a micromass cell pellet stained with Alcian blue, and collagen type II expression. Cells cultured in control culture media for 4 wk and stained for lineage markers are shown as insets (**D–G**) for each lineage and as (–) for RT-PCR analysis; *18S* mRNA was the internal control. Shown are results from a single patient sample representative of three (patient sample nos. 4, 5, 6, and 13). Bars = 50 μm (including insets).

undergo several rounds of proliferation to produce small clones [10], in a similar manner to that demonstrated for epidermal and hemopoietic stem cell hierarchies [1, 4].

Various human endometrial stromal populations can be induced to differentiate into one or more mesenchymal lineages when cultured under appropriate differentiation-inducing conditions [12, 15, 30]. In adipogenic or chondrogenic media, an undefined proportion of cells differentiated into adipocytes or chondrocytes [15, 30]. Furthermore, endometrial cells cultured from a single sample of menstrual blood also differentiated into five mesenchymal lineages and into a single neural lineage [36]. However, human endometrial stroma comprises a heterogeneous population of cells, and it is uncertain which cells are represented in these experiments. These studies highlight an unresolved question about whether nonclonogenic endometrial stromal cells are multipotent and have multilineage differentiation capacity. Only some of the stromal cells appeared to differentiate, and they may represent the 15% of clonogenic cells present in these cultures [30]. Studies on the MCAM $(CD146)^{+}PDGFR^{+}$ endometrial stromal cell population enriched 8 fold for stromal CFU, demonstrated that most cells differentiated into 4 mesenchymal lineages (adipogenic, myogenic, chondrogenic, and osteogenic) [12], while the MCAM $(CD146)^{-}PDGFR^{-}$ cells lacked this capacity, suggesting that the clonogenic cells were responsible for mesenchymal differentiation. In contrast, the present study demonstrates that large endometrial stromal CFU, which comprise 0.02% of fresh endometrial stromal cells [10], underwent multilineage differentiation at the single-cell level. More comprehensive quantitative studies are required to determine if a larger proportion of endometrial stromal cells differentiate into one or several lineages. However, such studies await the identification of a marker that enriches endometrial MSCs to purity, to allow for comparisons between prospectively isolated pure populations of MSCs and non-MSCs for multipotency.

Epithelial stem/progenitor cells have been identified in some human tissues with varying rates of cellular turnover, including rapidly proliferating epidermis [37], ocular surface [22] and intestine [38], occasionally remodelling breast epithelium [33], and relatively static prostate [39] epithelium. Human clonogenic epithelial cells typically produce several types of colony, which have different capacities for self-renewal, differentiation and proliferative potential. Similar to endometrial epithelial CFU, only the rarer and largest epidermal keratinocyte (holoclones) [37] and prostate epithelial [39] CFU demonstrated adult stem cell activity. A progressive loss of serial cloning activity and conversion to small CFU was observed for large endometrial epithelial tertiary and higher order CFU, similar to epidermal holoclones [37], suggesting progressive differentiation of self-renewing epithelial CFU. In contrast, the number of mammosphere CFU [33] remained constant over 5 serial cloning passages suggesting that sphere culturing is superior to 2D culture for maintaining self-renewing human epithelial stem/progenitor cells in vitro. It is currently unknown whether clonogenic endometrial epithelial cells can be cultured as spheres. The proliferative potential (cell generations) of large endometrial epithelial CFU (40) is 2- to 4-fold less than

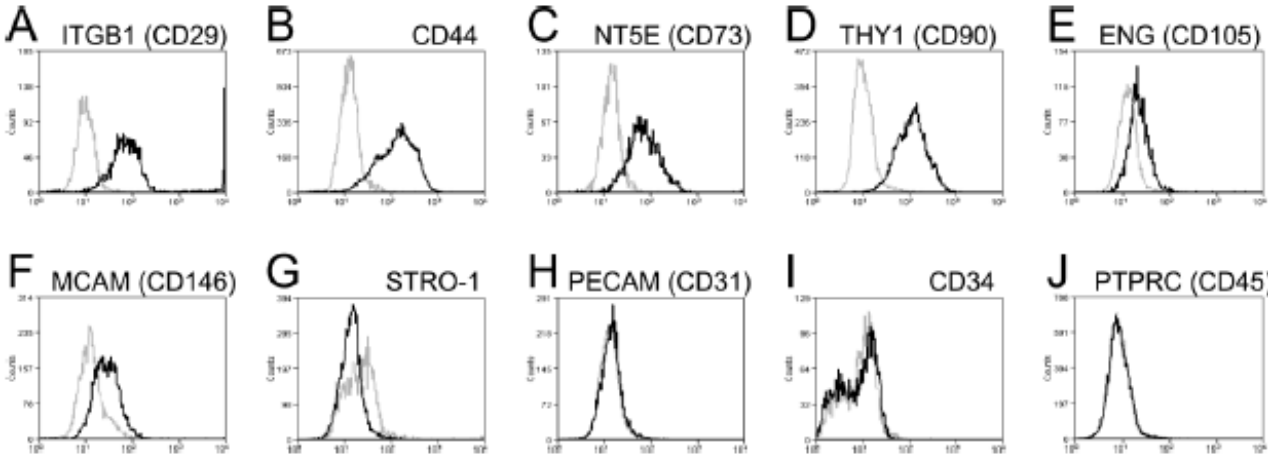

FIG. 5. Phenotyping of human endometrial stromal secondary CFU for typical MSC surface markers. (A–G) Single-parameter histograms for individual MSC markers and (H–J) MSC exclusion markers, representative of two to three patient samples (nos. 2, 4, and 5) (black lines). Gray lines indicate background fluorescence obtained with isotype control IgG or IgM. The x axis represents fluorescence intensity, and the y axis represents cell counts.

epidermal (120–160) [37] and ocular keratinocyte CFU (80–100) [22], reflecting differences in cellular turnover between the continuously proliferating epidermal tissues and cyclically regenerating endometrial epithelium, which undergoes rapid expansion in the first 7–10 days of each menstrual cycle [1]. Clonally derived endometrial epithelial CFU differentiated into cytokeratin-expressing gland-like structures likely mediated by endometrial stromal cell signals, that together with the Matrigel extracellular matrix provided a stem cell "niche" permissive for the differentiation into simple, pseudostratified epithelium that lines endometrial glands. Unlike epidermis, mammary gland or prostate there is no basal cell layer of a different cell phenotype, nor are several derivatives produced, like ocular goblet cells. Together our data suggest that human endometrium contains a rare epithelial progenitor that self renews, has high proliferative potential and is probably unipotential.

Multipotent mesenchymal stromal cells (MSCs), defined as plastic adherent with a characteristic surface phenotype capable of differentiating into osteoblasts, adipocytes, and chondroblasts in vitro [40], have been identified in several human tissues, including bone marrow [25], adipose [26], dental pulp [41], placenta, skeletal muscle, and pancreas [42], and, now, in endometrium. Similar to endometrial MSCs, colony size variation is a feature of bone marrow MSCs, with large CFU demonstrating defining MSC properties [43]. There are limited data on human MSC self-renewal, although it has been demonstrated in vivo for dental pulp MSCs [41] and in vitro for human bone marrow [44]. Large endometrial stromal CFU can be regarded as MSCs, as they generate quaternary clones, more than the minimum definition of adult stem cell status (secondary clones) [35]. As for hemopoietic stem cells [28], persisting endometrial CFU diminished with serial cloning, and at a slower rate for large compared with small endometrial stromal CFU. The extensive proliferative capacity of human endometrial stromal cells has been exploited to provide feeder layers for supporting human embryonic stem cell cell culture [45]. Similarly, large endometrial stromal CFU demonstrate substantial proliferative capacity (30 PDs), greater than most human bone marrow, dental pulp, and adipose CFU-F (<20 PDs) [41, 46, 47], which have similar capacity to that of small endometrial stromal CFU (13 PDs), and less than that of human fetal muscle cells (40 PDs) [42]. Thus, human endometrium contains a subpopulation of multipotent mesenchymal stromal cells that self-renew and have high proliferative potential, suggesting that they are MSCs. Similar to MSCs in other tissues [42, 48], human endometrial MSCs are found in a perivascular niche [12].

Importantly, previous data have demonstrated reconstitution of endometrial tissue from xenografted human endometrial cells [49]. Single-cell suspensions of unfractionated human endometrial cells injected directly beneath the kidney capsule of NOG mice generated endometrial tissue comprising glands that proliferated in response to estrogen, and stroma that differentiated into decidual cells on further administration of progesterone. Withdrawal of hormones resulted in a menstruation-like event in the reconstituted endometrial tissue [49]. Transplantation of human endometrial tissue fragments into immunocompromised mice has served as a mouse model for investigating endometriosis [50]. The survival and remodeling of the transplanted cells and tissue suggests that endometrial epithelial progenitors and MSCs, present in the original transplant, may be responsible for ectopic endometrial tissue growth. It also suggests their possible role in the pathophysiology of endometriosis [1]. It is important that future studies examine the capacity of clonally derived endometrial CFU to reconstitute endometrial tissue in vivo using similar xenotransplantation models as reported by Masuda et al. [49]. Consideration will need to be given to recapitulate stem cell niche conditions to enable transplanted stem/progenitor cells to generate endometrial tissue.

Our study has demonstrated adult stem cell activity for two cell types in human endometrium: an epithelial progenitor and an MSC. However, the stem cell assays used to identify these adult stem cells are retrospective, and do not allow their prospective isolation from endometrium, nor do they identify their location in endometrial tissue. We speculated that both endometrial epithelial and stromal CFU would be found in the basalis layer that remains during menstruation, and from which the new functionalis regenerates [1]. Interestingly, our recent discovery of markers that partially purify endometrial MSCs showed that they are located in a perivascular niche in both the functionalis and basalis blood vessels [12]. However, there are currently no known markers for endometrial epithelial progenitors, and their location is currently unknown.

Our demonstration of adult stem/progenitor cell and transit-amplifying cell activity in human endometrium provides the

impetus for discovery of endometrial epithelial stem/progenitor cell markers and more specific MSC markers to distinguish these cell types, as the assays that we have developed provide a means for assessing the effectiveness of potential markers [12]. It will then be possible to determine their expression of steroid hormone receptors, their location in normal endometrium, and to investigate the role of endometrial stem/progenitor cells in the pathogenesis of common gynecological diseases associated with abnormal endometrial regeneration. Such knowledge will enhance our understanding of endometriosis, adenomyosis, endometrial hyperplasia, and endometrial cancer, and has the potential to provide new therapeutic options targeting key endometrial stem/progenitor cell functions in preference to current hormonal manipulations. Endometrial MSCs may also provide an alternative readily available source of autologous MSCs for cell-based therapies in tissue engineering applications.

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Appendix 2 Genes with higher expression in Post-M *vs.* Pre-M human endometrial epithelial cells (P < 0.05 and fold change > 2)

Gene symbol	Gene description	P-value	Fold change
RARRES1	retinoic acid receptor responder (tazarotene induced) 1	0.005	12.90
HBB	hemoglobin, beta	0.023	12.72
HLA-DPA1	major histocompatibility complex, class II, DP alpha 1	0.026	12.08
S100A9	S100 calcium binding protein A9	0.027	11.83
S100A8	S100 calcium binding protein A8	0.003	11.43
ITGB2	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	0.004	10.94
LCN2	lipocalin 2	0.028	10.68
CFB	complement factor B	0.017	10.33
HBA2	hemoglobin, alpha 2	0.024	9.76
DMBT1	deleted in malignant brain tumors 1	0.031	9.48
HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1	0.020	9.13
HLA-DRA	major histocompatibility complex, class II, DR alpha	0.036	8.63
NFIX	nuclear factor I/X (CCAAT-binding transcription factor)	0.002	8.13
HLA-DRB1	major histocompatibility complex, class II, DR beta 1	0.040	7.94
MMP7	matrix metallopeptidase 7 (matrilysin, uterine)	0.015	7.91
KRT17	keratin 17	0.015	7.80
CD14	CD14 molecule	0.004	7.55
RGS1	regulator of G-protein signaling 1	0.026	7.20
ALOX5AP	arachidonate 5-lipoxygenase-activating protein	0.012	7.18
CDH3	cadherin 3, type 1, P-cadherin (placental)	0.012	7.12
FCGBP	Fc fragment of IgG binding protein	0.000	7.04
CD74	CD74 molecule, major histocompatibility complex, class II invariant chain	0.008	6.70
CSF1R	colony stimulating factor 1 receptor	0.010	6.70
UBD	ubiquitin D	0.029	6.59
C1QB	complement component 1, q subcomponent, B chain	0.032	6.58
SLCO2A1	solute carrier organic anion transporter family, member 2A1	0.010	6.48
C20ORF103	chromosome 20 open reading frame 103	0.020	6.41
CDH2	cadherin 2, type 1, N-cadherin (neuronal)	0.018	6.30
SCGB3A1	secretoglobin, family 3A, member 1	0.002	6.19
VTCN1	V-set domain containing T cell activation inhibitor 1	0.001	6.06
RBP1	retinol binding protein 1, cellular	0.016	6.04
DDIT4L	DNA-damage-inducible transcript 4-like	0.010	5.91
HP	haptoglobin	0.022	5.87
REG1A	regenerating islet-derived 1 alpha	0.029	5.86
TYROBP	TYRO protein tyrosine kinase binding protein	0.048	5.79
PHF15	PHD finger protein 15	0.000	5.62
HLA-DOA	major histocompatibility complex, class II, DO alpha	0.008	5.58
SCARA3	scavenger receptor class A, member 3	0.013	5.53
ATP6V1B1	ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B1	0.016	5.35
COL9A2	collagen, type IX, alpha 2	0.002	5.33
HLA-DRB3	major histocompatibility complex, class II, DR beta 3	0.003	5.20
OLR1	oxidized low density lipoprotein (lectin-like) receptor 1	0.003	5.16
LAPTM5	lysosomal protein transmembrane 5	0.006	5.04
C1QC	complement component 1, q subcomponent, C chain	0.037	4.94
STRA6	stimulated by retinoic acid gene 6 homolog (mouse)	0.006	4.92
FGF9	fibroblast growth factor 9 (glia-activating factor)	0.015	4.73
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	0.011	4.70

FBN2	fibrillin 2	0.018	4.69
RASL11B	RAS-like, family 11, member B	0.013	4.68
CH25H	cholesterol 25-hydroxylase	0.000	4.67
MMP11	matrix metallopeptidase 11 (stromelysin 3)	0.030	4.65
MSLN	mesothelin	0.025	4.52
IER5L	immediate early response 5-like	0.026	4.43
C10ORF81	chromosome 10 open reading frame 81	0.021	4.40
THBS4	thrombospondin 4	0.002	4.39
GPNMB	glycoprotein (transmembrane) nmb	0.030	4.34
SLC15A3	solute carrier family 15, member 3	0.034	4.33
CFTR	cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7)	0.014	4.19
CHURC1	churchill domain containing 1	0.000	4.19
SALL2	sal-like 2 (Drosophila)	0.001	4.17
TLE2	transducin-like enhancer of split 2 (E(sp1) homolog, Drosophila)	0.017	4.17
TMEM173	transmembrane protein 173	0.026	4.16
STK32B	serine/threonine kinase 32B	0.005	4.11
ATHL1	ATH1, acid trehalase-like 1 (yeast)	0.039	4.06
MATN2	matrilin 2	0.002	4.05
MS4A6A	membrane-spanning 4-domains, subfamily A, member 6A	0.004	4.00
VANGL2	vang-like 2 (van gogh, Drosophila)	0.002	3.96
FZD2	frizzled homolog 2 (Drosophila)	0.006	3.96
APCDD1	adenomatosis polyposis coli down-regulated 1	0.018	3.88
FAM20A	family with sequence similarity 20, member A	0.012	3.85
PLEK	pleckstrin	0.040	3.75
FMOD	fibromodulin	0.019	3.72
SNCAIP	synuclein, alpha interacting protein	0.034	3.70
IFI44L	interferon-induced protein 44-like	0.002	3.67
BCL11A	B-cell CLL/lymphoma 11A (zinc finger protein)	0.008	3.66
HLA-DPB1	major histocompatibility complex, class II, DP beta 1	0.009	3.65
BCAT1	branched chain amino-acid transaminase 1, cytosolic	0.005	3.59
SLC27A1	solute carrier family 27 (fatty acid transporter), member 1	0.005	3.59
HLA-DRB6	major histocompatibility complex, class II, DR beta 6 (pseudogene)	0.013	3.56
PROM2	prominin 2	0.020	3.51
DTX3	deltex homolog 3 (Drosophila)	0.001	3.48
RAB26	RAB26, member RAS oncogene family	0.042	3.48
SLCO2B1	solute carrier organic anion transporter family, member 2B1	0.002	3.48
GRAMD2	GRAM domain containing 2	0.001	3.48
BST2	bone marrow stromal cell antigen 2	0.006	3.45
MFAP2	microfibrillar-associated protein 2	0.004	3.41
C5ORF13	chromosome 5 open reading frame 13	0.040	3.41
ITGB4	integrin, beta 4	0.026	3.41
LEMD1	LEM domain containing 1	0.003	3.40
FCN1	ficolin (collagen/fibrinogen domain containing) 1	0.001	3.39
FHOD3	formin homology 2 domain containing 3	0.002	3.39
BZRAP1	enzodiazapine receptor (peripheral) associated protein 1	0.005	3.37
BEX2	brain expressed X-linked 2	0.001	3.37
TMEM98	transmembrane protein 98	0.036	3.34
VAV3	vav 3 guanine nucleotide exchange factor	0.004	3.33
CYP4F12	cytochrome P450, family 4, subfamily F, polypeptide 12	0.032	3.30
PLA2G7	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	0.046	3.30

LRRN2	leucine rich repeat neuronal 2	0.000	3.28
TNFRSF19	tumor necrosis factor receptor superfamily, member 19	0.001	3.28
PARVG	parvin, gamma	0.007	3.27
CAPN13	calpain 13	0.030	3.27
XAF1	XIAP associated factor 1	0.002	3.21
FAM20C	family with sequence similarity 20, member C	0.020	3.21
AXIN2	axin 2	0.017	3.20
ACOX2	acyl-CoA oxidase 2, branched chain	0.001	3.15
SLC13A5	solute carrier family 13 (sodium-dependent citrate transporter), member 5	0.045	3.14
CCDC102A	coiled-coil domain containing 102A	0.018	3.13
IFITM1	interferon induced transmembrane protein 1 (9-27)	0.029	3.11
NFE2	nuclear factor (erythroid-derived 2), 45kDa	0.007	3.10
ARNT2	aryl-hydrocarbon receptor nuclear translocator 2	0.019	3.09
C1ORF188	ring finger protein 207	0.009	3.08
LEF1	lymphoid enhancer-binding factor 1	0.005	3.04
RNASE1	ribonuclease, RNase A family, 1 (pancreatic)	0.023	3.03
EGR2	early growth response 2	0.016	3.02
C15ORF52	chromosome 15 open reading frame 52	0.033	3.02
PCDHB2	protocadherin beta 2	0.010	3.01
SOX9	SRY (sex determining region Y)-box 9	0.004	2.99
CTSF	cathepsin F	0.009	2.97
BRSK1	BR serine/threonine kinase 1	0.020	2.97
ACACB	acetyl-CoA carboxylase beta	0.005	2.95
C1QTNF5	C1q and tumor necrosis factor related protein 5	0.047	2.95
BEX1	brain expressed, X-linked 1	0.018	2.95
PYCARD	PYD and CARD domain containing	0.012	2.94
C14ORF152	family with sequence similarity 181, member A	0.012	2.94
ALDH1L1	aldehyde dehydrogenase 1 family, member L1	0.015	2.94
KIAA0644	TLR4 interactor with leucine-rich repeats	0.000	2.92
LTB	lymphotoxin beta (TNF superfamily, member 3)	0.033	2.91
ALDH3A1	aldehyde dehydrogenase 3 family, member A1	0.003	2.90
FGD3	FYVE, RhoGEF and PH domain containing 3	0.008	2.90
TESC	tescalcin	0.009	2.87
ETV4	ets variant 4	0.001	2.87
ROR2	receptor tyrosine kinase-like orphan receptor 2	0.005	2.86
GGT6	gamma-glutamyltransferase 6	0.018	2.84
LRRTM1	leucine rich repeat transmembrane neuronal 1	0.007	2.84
PDE7B	phosphodiesterase 7B	0.000	2.83
RNF165	ring finger protein 165	0.006	2.83
VWA1	von Willebrand factor A domain containing 1	0.005	2.82
OSTALPHA	organic solute transporter alpha	0.015	2.81
OGDHL	oxoglutarate dehydrogenase-like	0.025	2.80
DACT2	dapper, antagonist of beta-catenin, homolog 2 (Xenopus laevis)	0.016	2.80
EPHB4	EPH receptor B4	0.019	2.77
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha	0.001	2.77
ZNF219	zinc finger protein 219	0.004	2.76
RASSF5	Ras association (RalGDS/AF-6) domain family member 5	0.027	2.76
EFNB3	ephrin-B3	0.000	2.75
SORL1	sortilin-related receptor, L(DLR class) A repeats containing	0.003	2.72
AIF1	allograft inflammatory factor 1	0.006	2.71
SCPEP1	serine carboxypeptidase 1	0.033	2.69
SLC16A9	solute carrier family 16, member 9 (monocarboxylic acid	0.015	2.69

	transporter 9)		
FLJ10781	PNMA-like 1	0.002	2.68
GALM	galactose mutarotase (aldose 1-epimerase)	0.038	2.67
MFGE8	milk fat globule-EGF factor 8 protein	0.030	2.66
WFDC2	WAP four-disulfide core domain 2	0.001	2.65
APBB3	amyloid beta (A4) precursor protein-binding, family B, member 3	0.010	2.65
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	0.006	2.64
RXRA	retinoid X receptor, alpha	0.030	2.61
SMARCD3	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 3	0.007	2.60
ADCY4	adenylate cyclase 4	0.036	2.60
NLRP3	NLR family, pyrin domain containing 3	0.004	2.60
ST6GALNAC2	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 2	0.002	2.60
CBX7	chromobox homolog 7	0.024	2.59
PLAC8	placenta-specific 8	0.012	2.59
FGF18	fibroblast growth factor 18	0.001	2.57
TEAD2	TEA domain family member 2	0.017	2.55
MEIS1	Meis homeobox 1	0.001	2.54
NOTUM	notum pectinacetylesterase homolog (Drosophila)	0.000	2.54
SYT17	synaptotagmin XVII	0.005	2.54
ACPL2	acid phosphatase-like 2	0.006	2.52
C1RL	complement component 1, r subcomponent-like	0.045	2.51
FAM50B	family with sequence similarity 50, member B	0.009	2.50
ARHGEF17	Rho guanine nucleotide exchange factor (GEF) 17	0.007	2.49
INPP5E	inositol polyphosphate-5-phosphatase, 72 kDa	0.008	2.48
ZNF337	zinc finger protein 337	0.004	2.47
ZNF533	zinc finger protein 385B	0.047	2.46
B3GNT6	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 6 (core 3 synthase)	0.012	2.46
OSBPL5	oxysterol binding protein-like 5	0.036	2.45
CCL4L1	chemokine (C-C motif) ligand 4-like 1	0.031	2.45
DACH1	dachshund homolog 1 (Drosophila)	0.006	2.45
FLJ45244	hypothetical locus FLJ45244	0.014	2.44
LRP5	low density lipoprotein receptor-related protein 5	0.002	2.41
WDR27	WD repeat domain 27	0.016	2.39
RPPH1	ribonuclease P RNA component H1	0.044	2.39
C22ORF29	chromosome 22 open reading frame 29	0.010	2.39
FCER1A	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide	0.005	2.38
NFE2L3	nuclear factor (erythroid-derived 2)-like 3	0.005	2.37
LOC388692	hypothetical LOC388692	0.034	2.37
FOXJ1	forkhead box J1	0.011	2.36
GPR132	G protein-coupled receptor 132	0.004	2.36
SNX33	sorting nexin 33	0.008	2.35
IFI44	interferon-induced protein 44	0.018	2.34
PTP4A3	protein tyrosine phosphatase type IVA, member 3	0.012	2.33
MGC42367	chromosome 2 open reading frame 55	0.009	2.33
LY86	lymphocyte antigen 86	0.002	2.33
PCGF2	polycomb group ring finger 2	0.003	2.33
UBA7	ubiquitin-like modifier activating enzyme 7	0.028	2.31
ZNF503	zinc finger protein 503	0.027	2.31

CFI	complement factor I	0.041	2.31
SMYD4	SET and MYND domain containing 4	0.006	2.31
RAB36	RAB36, member RAS oncogene family	0.011	2.30
HLA-DQB2	major histocompatibility complex, class II, DQ beta 2	0.014	2.30
NAV1	neuron navigator 1	0.008	2.29
MGC24103	hypothetical MGC24103	0.009	2.29
C5ORF4	chromosome 5 open reading frame 4	0.000	2.28
NOTCH4	notch 4	0.017	2.28
INPP5D	inositol polyphosphate-5-phosphatase, 145kDa	0.026	2.28
STOX2	storkhead box 2	0.032	2.27
KLHDC8B	kelch domain containing 8B	0.038	2.27
NLGN4X	neuroligin 4, X-linked	0.027	2.26
PLXNB1	plexin B1	0.022	2.26
ZNF385A	zinc finger protein 385A	0.014	2.26
ZNF362	zinc finger protein 362	0.006	2.25
TNFSF9	tumor necrosis factor (ligand) superfamily, member 9	0.040	2.24
LYPD1	LY6/PLAUR domain containing 1	0.009	2.24
ZFP90	zinc finger protein 90 homolog (mouse)	0.011	2.24
NKD1	naked cuticle homolog 1 (Drosophila)	0.012	2.24
SLC22A17	solute carrier family 22, member 17	0.033	2.24
DOCK2	dedicator of cytokinesis 2	0.023	2.23
NISCH	nischarin	0.017	2.23
TNFRSF25	tumor necrosis factor receptor superfamily, member 25	0.026	2.23
CRYBB2	crystallin, beta B2	0.010	2.23
AUTS2	autism susceptibility candidate 2	0.026	2.23
ZNF692	zinc finger protein 692	0.015	2.23
GSTM3	glutathione S-transferase mu 3 (brain)	0.014	2.22
FLJ39653	hypothetical FLJ39653	0.010	2.20
RBM38	RNA binding motif protein 38	0.044	2.19
LGALS9	lectin, galactoside-binding, soluble, 9	0.014	2.19
IDUA	iduronidase, alpha-L-	0.025	2.18
FABP6	fatty acid binding protein 6, ileal	0.046	2.18
LOC390876	ribosomal protein L35 pseudogene	0.002	2.18
WDR86	WD repeat domain 86	0.012	2.18
ASCL2	achaete-scute complex homolog 2 (Drosophila)	0.036	2.17
CMTM7	CKLF-like MARVEL transmembrane domain containing 7	0.007	2.17
HLA-DQB1	major histocompatibility complex, class II, DQ beta 1	0.042	2.17
TBPL2	TATA box binding protein like 2	0.005	2.17
C3ORF15	chromosome 3 open reading frame 15	0.007	2.17
MTMR15	FANCD2/FANCI-associated nuclease 1	0.003	2.16
ZNF500	zinc finger protein 500	0.020	2.16
WNT10A	wingless-type MMTV integration site family, member 10A	0.032	2.15
FGD1	FYVE, RhoGEF and PH domain containing 1	0.000	2.15
H6PD	hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase)	0.002	2.15
EPHX2	epoxide hydrolase 2, cytoplasmic	0.030	2.15
C20ORF196	chromosome 20 open reading frame 196	0.004	2.14
PLEKHA4	pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 4	0.023	2.14
KIAA1787	neuralized homolog 4 (Drosophila)	0.023	2.14
D4S234E	DNA segment on chromosome 4 (unique) 234 expressed sequence	0.049	2.14
TUBG2	tubulin, gamma 2	0.004	2.14
FPR1	formyl peptide receptor 1	0.001	2.13

ZNF177	zinc finger protein 177	0.029	2.13
GADD45G	growth arrest and DNA-damage-inducible, gamma	0.017	2.13
FLJ14213	proline rich 5 like	0.003	2.13
TSEN54	tRNA splicing endonuclease 54 homolog (S. cerevisiae)	0.041	2.13
ZNF133	zinc finger protein 133	0.011	2.12
IFT140	intraflagellar transport 140 homolog (Chlamydomonas)	0.025	2.12
FRS3	fibroblast growth factor receptor substrate 3	0.019	2.11
PGM5	phosphoglucomutase 5	0.022	2.11
SBK1	SH3-binding domain kinase 1	0.043	2.11
TSPAN6	tetraspanin 6	0.030	2.11
C22ORF36	chromosome 22 open reading frame 36	0.033	2.10
CLCNKA	chloride channel Ka	0.014	2.10
LAPTM4B	lysosomal protein transmembrane 4 beta	0.025	2.08
ZNF444	zinc finger protein 444	0.012	2.07
GBP4	guanylate binding protein 4	0.034	2.07
SMO	smoothened homolog (Drosophila)	0.015	2.06
IRAK1BP1	interleukin-1 receptor-associated kinase 1 binding protein 1	0.001	2.06
CHST12	carbohydrate (chondroitin 4) sulfotransferase 12	0.006	2.06
GAA	glucosidase, alpha; acid	0.040	2.06
FCGRT	Fc fragment of IgG, receptor, transporter, alpha	0.015	2.05
ZNF827	zinc finger protein 827	0.036	2.05
CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	0.018	2.04
ALDH3B1	aldehyde dehydrogenase 3 family, member B1	0.030	2.04
TEX2	testis expressed 2	0.005	2.02
NAGPA	N-acetylglucosamine-1-phosphodiester alpha-N-acetylglucosaminidase	0.039	2.01
MED12	mediator complex subunit 12	0.009	2.01
LTBP3	latent transforming growth factor beta binding protein 3	0.028	2.01
TEF	thyrotrophic embryonic factor	0.041	2.01

Appendix 3 Focus genes for selected enriched gene ontology (GO) classifications of highly expressed genes in Post-M *vs.* Pre-M endometrial epithelial cells

Enriched GO gene families	Fisher Exact test	Focus Genes
Biological processes		
Immune response	2.84E-08	HLA-DQB1, HLA-DRB1, VTCN1, LY86, HLA-DRB3, IFI44L, FCGRT, C1QC, CD74, TMEM173, FCN1, INPP5D, HLA-DPB1, CFI, HLA-DOA, LTB, BST2, OLR1, FOXJ1, CFB, CCL4L1, NLRP3, TNFSF9, HLA-DQA1, C1QB, TNFSF10, RGS1, C1RL, HLA-DPA1, GBP4, CD14, HLA-DRA, DMBT1
Response to wounding	2.39E-04	OLR1, S100A8, PLEK, AIF1, CFB, RXRA, LY86, S100A9, EPHX2, CCL4L1, ITGB2, CDH3, NLRP3, C1QC, SMO, C1QB, C1RL, PLA2G7, CFI, SYT17, CD14, WFDC2
Regulation of cell proliferation	1.27E-03	FGF18, RARRES1, IFITM1, FGF9, AIF1, ARNT2, MMP7, SOX9, REG1A, INPP5D, AXIN2, GPNMB, LTB, CEBPA, TESC, FOXJ1, RXRA, MFGE8, CDKN1C, SMO, NOTCH4, ADAMTS1, SCGB3A1, FABP6, LRP5, DMBT1, SMO, VANGL2
Apoptosis	2.80E-03	FGD1, VAV3, TNFRSF25, LY86, ARHGEF17, ITGB2, NLRP3, TNFSF9, RASSF5, TNFSF10, TMEM173, NISCH, GADD45G, PYCARD, TNFRSF19, XAF1, INPP5D, LTB, FGD3, CD14
Wnt receptor signalling pathway	4.83E-03	WNT10A, NKD1, LEF1, ROR2, TLE2, FZD2, AXIN2, LRP5
Cell adhesion, recognition, migration	1.34E-02	PARVG, PLEK, OLR1, LRRN2, ITGB4, PCDHB2, CCL4L1, LEF1, MFGE8, ITGB2, CDH3, SOX9, PGM5, MSLN, NLGN4X, ROR2, FCGBP, GPNMB, THBS4, DOCK2, MFGE8, FCGBP, SMO, VAV3, PLXNB1, FOXJ1, S100A9, CDH2, THBS4, LRP5
Intracellular signalling cascade	8.27E-02	ADCY4, FPR1, BRSK1, CD74, NISCH, TNFRSF19, RAB26, INPP5D, LTB, TYROBP, FCER1A, VAV3, PLEK, PLXNB1, RXRA, MED12, FZD2, RASL11B, RASSF5, RGS1, RAB36, GADD45G, NOTCH4, ROR2, RBM38, APBB3, IRAK1BP1
Cell-cell signalling	8.69E-02	FCER1A, FGF18, EFNB3, BST2, FGF9, S100A9, PCDHB2, CCL4L1, ITGB2, FZD2, TNFSF9, SMO, PDE7B, TNFSF10, LTB
Steroid metabolic process	9.68E-02	ACOX2, OSBPL5, CH25H, RXRA, SORL1, CFTR, FABP6
Molecular functions		
MHC class II receptor activity	5.19E-09	HLA-DQB1, HLA-DRB1, HLA-DRB3, HLA-DPA1, HLA-DPB1, HLA-DOA, HLA-DQA1, HLA-DRA
Lipid binding	1.40E-02	ACOX2, OSBPL5, VAV3, PLEK, RBP1, RXRA, MFGE8, PLEKHA4, RASSF5, NISCH, ALOX5AP, PLA2G7, SNX33, FABP6
Carbohydrate binding	1.41E-02	GALM, REG1A, OLR1, FGF9, FCN1, GAA, ADAMTS1, GPNMB, NLRP3, LGALS9, CD14, THBS4
Transcription factor activity	2.51E-02	CEBPA, EGR2, NFE2, FOXJ1, ZNF133, RXRA, ARNT2, LEF1, TEAD2, NFIX, DACH1, SOX9, MEIS1, ZNF500, ASCL2, TBPL2, SALL2, PCGF2, TEF, ZNF444, NFE2L3, ZNF219, ETV4
Cellular components		

MHC protein complex	1.97E-06	HLA-DQB1, HLA-DRB1, HLA-DRB3, FCGRT, HLA-DPA1, HLA-DPB1, HLA-DOA, HLA-DQA1, HLA-DRA
extracellular region	2.88E-04	SCPEP1, FGF18, LTBP3, FAM20A, FGF9, LY86, FAM20C, SORL1, MMP7, NOTUM, HP, C1QC, CFI, VWA1, LTB, MATN2, WNT10A, PLXNB1, CCL4L1, MFGE8, MMP11, C1QB, PLA2G7, MFAP2, ADAMTS1, SCGB3A1, WFDC2, FMOD, TNFRSF25, C1QTNF5, COL9A2, FCN1, MSLN, FBN2, THBS4, RNASE1, PLEK, OLR1, CFB, TNFSF9, LGALS9, LCN2, TNFSF10, ACPL2, NOTCH4, C1RL, CMTM7, FCGBP, CD14, DMBT1
integral to plasma membrane	3.25E-02	HLA-DRB1, TNFRSF25, HLA-DRB3, SORL1, CLCNKA, ITGB4, ITGB2, EPHB4, SLCO2A1, LAPTM5, GPNMB, TYROBP, CSF1R, FCER1A, EFNB3, OLR1, BST2, PLXNB1, PCDHB2, FZD2, HLA-DQA1, TNFSF10, NOTCH4, NLGN4X, ROR2, HLA-DPA1, HLA-DRA

This table was generated by DAVID functional classification tool from the high expression gene list (Appendix 2).

Appendix 4 Genes with low expression in Post-M *vs.* Pre-M human endometrial epithelial cells (P < 0.05 and fold change > 2)

Gene symbol	Gene description	P-value	Fold change
SCGB1D4	secretoglobin, family 1D, member 4	0.002	-90.07
SCGB1D2	secretoglobin, family 1D, member 2	0.005	-31.11
HSD17B2	hydroxysteroid (17-beta) dehydrogenase 2	0.001	-26.88
SLC18A2	solute carrier family 18 (vesicular monoamine), member 2	0.000	-18.03
BAIAP2L2	BAI1-associated protein 2-like 2	0.001	-14.41
C6ORF141	chromosome 6 open reading frame 141	0.000	-13.86
SLC3A1	solute carrier family 3 (cystine, dibasic and neutral amino acid transporters, activator of cystine, dibasic and neutral amino acid transport), member 1	0.004	-13.82
CD36	CD36 molecule (thrombospondin receptor)	0.001	-9.92
APOBEC3B	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B	0.003	-9.86
GSTT1	glutathione S-transferase theta 1	0.006	-9.56
VNN1	vanin 1	0.006	-9.47
ATP6V1C2	ATPase, H+ transporting, lysosomal 42kDa, V1 subunit C2	0.000	-9.30
SLC30A2	solute carrier family 30 (zinc transporter), member 2	0.004	-8.56
SPHK1	sphingosine kinase 1	0.000	-8.11
ACPP	acid phosphatase, prostate	0.001	-8.09
USP53	ubiquitin specific peptidase 53	0.003	-8.03
RASGRP3	RAS guanyl releasing protein 3 (calcium and DAG-regulated)	0.012	-7.82
RHOBTB3	Rho-related BTB domain containing 3	0.002	-7.73
GCNT3	glucosaminyl (N-acetyl) transferase 3, mucin type	0.016	-7.72
HLA-DOB	major histocompatibility complex, class II, DO beta	0.047	-7.59
MT1G	metallothionein 1G	0.001	-7.57
COL1A2	collagen, type I, alpha 2	0.009	-7.56
ZBTB43	zinc finger and BTB domain containing 43	0.001	-7.25
ISG20	interferon stimulated exonuclease gene 20kDa	0.000	-7.22
CST1	cystatin SN	0.010	-7.21
MT1H	metallothionein 1H	0.000	-6.95
SDCBP2	syndecan binding protein (syntenin) 2	0.001	-6.92
F2RL1	coagulation factor II (thrombin) receptor-like 1	0.002	-6.71
IDH1	isocitrate dehydrogenase 1 (NADP+), soluble	0.038	-6.38
ITGA2	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	0.036	-6.28
AGPAT5	1-acylglycerol-3-phosphate O-acyltransferase 5 (lysophosphatidic acid acyltransferase, epsilon)	0.001	-6.05
PPARG	peroxisome proliferator-activated receptor gamma	0.005	-6.01
SCGB2A2	secretoglobin, family 2A, member 2	0.040	-6.00
PPTC7	PTC7 protein phosphatase homolog (S. cerevisiae)	0.001	-5.97
RXFP1	relaxin/insulin-like family peptide receptor 1	0.016	-5.95
MGST1	microsomal glutathione S-transferase 1	0.002	-5.77
SLC39A14	solute carrier family 39 (zinc transporter), member 14	0.003	-5.76
RASEF	RAS and EF-hand domain containing	0.006	-5.73
TXNRD1	thioredoxin reductase 1	0.030	-5.69
C1ORF71	consortin, connexin sorting protein	0.006	-5.61
SESTD1	SEC14 and spectrin domains 1	0.001	-5.57
LIPG	lipase, endothelial	0.037	-5.57
SMS	spermine synthase	0.000	-5.43
CKMT2	creatine kinase, mitochondrial 2 (sarcomeric)	0.014	-5.42
ACTG2	actin, gamma 2, smooth muscle, enteric	0.036	-5.42
DENND2C	DENN/MADD domain containing 2C	0.003	-5.34

SPATA13	spermatogenesis associated 13	0.013	-5.28
BCL10	B-cell CLL/lymphoma 10	0.001	-5.27
ADFP	perilipin 2	0.001	-5.27
SBSN	suprabasin	0.012	-5.23
HGD	homogentisate 1,2-dioxygenase	0.006	-5.20
CABLES1	Cdk5 and Abl enzyme substrate 1	0.000	-5.16
OTUB2	OTU domain, ubiquitin aldehyde binding 2	0.005	-4.96
PLAUR	plasminogen activator, urokinase receptor	0.003	-4.92
TFPI2	tissue factor pathway inhibitor 2	0.015	-4.88
STEAP4	STEAP family member 4	0.028	-4.87
VCAN	versican	0.018	-4.86
GNG11	guanine nucleotide binding protein (G protein), gamma 11	0.040	-4.82
RYBP	RING1 and YY1 binding protein	0.026	-4.79
C3ORF52	chromosome 3 open reading frame 52	0.008	-4.79
MRPL44	mitochondrial ribosomal protein L44	0.006	-4.75
SFN	stratifin	0.030	-4.74
SSFA2	sperm specific antigen 2	0.002	-4.74
C1ORF116	chromosome 1 open reading frame 116	0.014	-4.65
TPM3	tropomyosin 3	0.003	-4.64
LRRFIP2	leucine rich repeat (in FLII) interacting protein 2	0.001	-4.64
STK40	serine/threonine kinase 40	0.001	-4.63
CTSL2	cathepsin L2	0.005	-4.62
GLRX	glutaredoxin (thioltransferase)	0.005	-4.62
ZNF217	zinc finger protein 217	0.005	-4.56
PAPSS1	3'-phosphoadenosine 5'-phosphosulfate synthase 1	0.005	-4.56
DUSP14	dual specificity phosphatase 14	0.003	-4.54
DYNLT3	dynein, light chain, Tctex-type 3	0.001	-4.53
KMO	kynurenine 3-monooxygenase (kynurenine 3-hydroxylase)	0.025	-4.51
ALDH6A1	aldehyde dehydrogenase 6 family, member A1	0.004	-4.49
CD55	CD55 molecule, decay accelerating factor for complement (Cromer blood group)	0.000	-4.46
SLC39A8	solute carrier family 39 (zinc transporter), member 8	0.004	-4.45
NPAL1	NIPA-like domain containing 1	0.020	-4.45
KCNG3	potassium voltage-gated channel, subfamily G, member 3	0.001	-4.44
C20ORF45	GNAS complex locus	0.002	-4.43
TBC1D23	TBC1 domain family, member 23	0.006	-4.39
UGCG	UDP-glucose ceramide glucosyltransferase	0.019	-4.38
SEC14L1	SEC14-like 1 (S. cerevisiae)	0.001	-4.33
SERPINB8	serpin peptidase inhibitor, clade B (ovalbumin), member 8	0.007	-4.31
LPIN1	lipin 1	0.020	-4.28
SLC7A2	solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	0.026	-4.19
FAM134B	family with sequence similarity 134, member B	0.033	-4.18
MGLL	monoglyceride lipase	0.001	-4.18
LAMA2	laminin, alpha 2	0.009	-4.15
OBFC2A	oligonucleotide/oligosaccharide-binding fold containing 2A	0.002	-4.15
ATL3	atlastin GTPase 3	0.001	-4.11
TLE1	transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)	0.010	-4.11
ZBTB38	zinc finger and BTB domain containing 38	0.011	-4.11
MCCC2	methylcrotonoyl-CoA carboxylase 2 (beta)	0.000	-4.08
TAP2	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	0.012	-4.07
CKB	creatine kinase, brain	0.037	-4.05

PGM3	phosphoglucomutase 3	0.008	-4.02
EHF	ets homologous factor	0.017	-4.01
ACSL3	acyl-CoA synthetase long-chain family member 3	0.004	-3.99
LARP2	La ribonucleoprotein domain family, member 1B	0.001	-3.98
EDN1	endothelin 1	0.001	-3.98
RIPK2	receptor-interacting serine-threonine kinase 2	0.002	-3.98
PLEKHB2	pleckstrin homology domain containing, family B (evectins) member 2	0.002	-3.97
GALNT4	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 4 (GalNAc-T4)	0.001	-3.94
NKIRAS1	NFKB inhibitor interacting Ras-like 1	0.012	-3.92
RGS20	regulator of G-protein signaling 20	0.023	-3.91
HEY1	hairy/enhancer-of-split related with YRPW motif 1	0.013	-3.90
ZC3HAV1	zinc finger CCCH-type, antiviral 1	0.001	-3.90
LEFTY1	left-right determination factor 1	0.046	-3.88
MFSD2	major facilitator superfamily domain containing 2A	0.003	-3.88
ACSM3	acyl-CoA synthetase medium-chain family member 3	0.004	-3.86
IL6R	interleukin 6 receptor	0.003	-3.85
ABHD3	abhydrolase domain containing 3	0.010	-3.84
ADAM9	ADAM metallopeptidase domain 9	0.008	-3.84
TNFRSF10A	tumor necrosis factor receptor superfamily, member 10a	0.024	-3.83
KIAA1324	KIAA1324	0.018	-3.81
TICAM1	toll-like receptor adaptor molecule 1	0.000	-3.80
ITGB8	integrin, beta 8	0.005	-3.76
C1ORF55	chromosome 1 open reading frame 55	0.024	-3.76
CATSPERB	cation channel, sperm-associated, beta	0.022	-3.75
ZFAND6	zinc finger, AN1-type domain 6	0.026	-3.74
CCNC	cyclin C	0.011	-3.73
QPRT	quinolinate phosphoribosyltransferase	0.016	-3.71
TMEM189	transmembrane protein 189	0.001	-3.68
FAM80B	ribosomal modification protein rimK-like family member B	0.025	-3.68
ZNF562	zinc finger protein 562	0.013	-3.67
KDELR2	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2	0.004	-3.67
HOOK1	hook homolog 1 (Drosophila)	0.013	-3.66
DAB2	disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)	0.014	-3.66
FAM84B	family with sequence similarity 84, member B	0.001	-3.66
KCNG1	potassium voltage-gated channel, subfamily G, member 1	0.045	-3.66
ARID3B	AT rich interactive domain 3B (BRIGHT-like)	0.033	-3.64
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase	0.015	-3.64
GCLM	glutamate-cysteine ligase, modifier subunit	0.039	-3.64
PECI	enoyl-CoA delta isomerase 2	0.018	-3.62
EIF2C2	eukaryotic translation initiation factor 2C, 2	0.012	-3.62
GADD45A	growth arrest and DNA-damage-inducible, alpha	0.001	-3.61
ISG20L1	apoptosis enhancing nuclease	0.004	-3.61
OKL38	oxidative stress induced growth inhibitor 1	0.002	-3.60
RAB11FIP1	RAB11 family interacting protein 1 (class I)	0.001	-3.59
NPC1	Niemann-Pick disease, type C1	0.005	-3.59
PPP3CC	protein phosphatase 3, catalytic subunit, gamma isozyme	0.000	-3.58
SQLE	squalene epoxidase	0.046	-3.58
PRKX	protein kinase, X-linked	0.034	-3.58
CCRN4L	CCR4 carbon catabolite repression 4-like (S. cerevisiae)	0.010	-3.57
ITPKC	inositol 1,4,5-trisphosphate 3-kinase C	0.000	-3.57

EDNRB	endothelin receptor type B	0.021	-3.53
SPAG9	sperm associated antigen 9	0.019	-3.53
RPS6KA5	ribosomal protein S6 kinase, 90kDa, polypeptide 5	0.006	-3.53
KRT13	keratin 13	0.004	-3.53
JAK1	Janus kinase 1	0.031	-3.52
KCNF1	potassium voltage-gated channel, subfamily F, member 1	0.033	-3.52
NUSAP1	nucleolar and spindle associated protein 1	0.021	-3.51
SPTBN1	spectrin, beta, non-erythrocytic 1	0.004	-3.50
GARS	glycyl-tRNA synthetase	0.010	-3.50
GINS3	GINS complex subunit 3 (Psf3 homolog)	0.005	-3.49
YOD1	YOD1 OTU deubiquinating enzyme 1 homolog (S. cerevisiae)	0.000	-3.49
PSMC4	proteasome (prosome, macropain) 26S subunit, ATPase, 4	0.005	-3.48
SLC38A1	solute carrier family 38, member 1	0.009	-3.48
CYB5R2	cytochrome b5 reductase 2	0.032	-3.48
TMOD1	tropomodulin 1	0.032	-3.48
SC4MOL	sterol-C4-methyl oxidase-like	0.019	-3.47
PCCA	propionyl CoA carboxylase, alpha polypeptide	0.003	-3.45
FGB	fibrinogen beta chain	0.015	-3.43
MPHOSPH10	M-phase phosphoprotein 10 (U3 small nucleolar ribonucleoprotein)	0.018	-3.42
TSPAN13	tetraspanin 13	0.008	-3.39
MT1F	metallothionein 1F	0.003	-3.38
RAD21	RAD21 homolog (S. pombe)	0.016	-3.37
B3GNT2	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 2	0.036	-3.36
POU5F1P1	POU class 5 homeobox 1B	0.027	-3.35
C5ORF23	natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C)	0.040	-3.35
OVGP1	oviductal glycoprotein 1, 120kDa	0.010	-3.34
BCAP29	B-cell receptor-associated protein 29	0.002	-3.31
WTAP	Wilms tumor 1 associated protein	0.004	-3.31
NRIP1	nuclear receptor interacting protein 1	0.010	-3.30
LGR4	leucine-rich repeat containing G protein-coupled receptor 4	0.006	-3.29
ALCAM	activated leukocyte cell adhesion molecule	0.006	-3.28
MTHFD2	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase	0.033	-3.28
MTHFD2L	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2-like	0.015	-3.28
TMEM189-UBE2V1	TMEM189-UBE2V1 readthrough	0.002	-3.28
MAP2K1	mitogen-activated protein kinase kinase 1	0.010	-3.27
LRRC31	leucine rich repeat containing 31	0.012	-3.26
WDR51B	POC1 centriolar protein homolog B (Chlamydomonas)	0.005	-3.25
MORF4L2	mortality factor 4 like 2	0.012	-3.25
PIGA	phosphatidylinositol glycan anchor biosynthesis, class A	0.002	-3.25
SPINK1	serine peptidase inhibitor, Kazal type 1	0.012	-3.24
GJB1	gap junction protein, beta 1, 32kDa	0.023	-3.24
PSME4	proteasome (prosome, macropain) activator subunit 4	0.002	-3.24
BMP6	bone morphogenetic protein 6	0.026	-3.24
SPOCD1	SPOC domain containing 1	0.006	-3.23
STIL	SCL/TAL1 interrupting locus	0.020	-3.22
ZCCHC6	zinc finger, CCHC domain containing 6	0.001	-3.22
CD99	CD99 molecule	0.001	-3.21
NLF2	C2 calcium-dependent domain containing 4B	0.027	-3.21

RAB9A	RAB9A, member RAS oncogene family	0.007	-3.20
LIMS1	LIM and senescent cell antigen-like domains 1	0.012	-3.20
USP38	ubiquitin specific peptidase 38	0.023	-3.19
PYGL	phosphorylase, glycogen, liver	0.004	-3.15
PGAM1	phosphoglycerate mutase 1 (brain)	0.021	-3.14
JOSD1	Josephin domain containing 1	0.005	-3.13
ATG4B	ATG4 autophagy related 4 homolog B (S. cerevisiae)	0.003	-3.13
C2ORF30	endoplasmic reticulum lectin 1	0.007	-3.13
SLC28A3	solute carrier family 28 (sodium-coupled nucleoside transporter), member 3	0.040	-3.11
ANKRD12	ankyrin repeat domain 12	0.006	-3.10
CRIM1	cysteine rich transmembrane BMP regulator 1 (chordin-like)	0.047	-3.10
CLDND1	claudin domain containing 1	0.004	-3.10
HPRT1	hypoxanthine phosphoribosyltransferase 1	0.048	-3.10
C14ORF147	chromosome 14 open reading frame 147	0.002	-3.10
ARID5B	AT rich interactive domain 5B (MRF1-like)	0.001	-3.08
SLC4A7	solute carrier family 4, sodium bicarbonate cotransporter, member 7	0.006	-3.08
PTP4A1	protein tyrosine phosphatase type IVA, member 1	0.001	-3.08
EAF2	ELL associated factor 2	0.030	-3.07
UBE2H	ubiquitin-conjugating enzyme E2H (UBC8 homolog, yeast)	0.013	-3.07
FAM148A	C2 calcium-dependent domain containing 4A	0.012	-3.06
DUSP4	dual specificity phosphatase 4	0.021	-3.06
EID3	EP300 interacting inhibitor of differentiation 3	0.030	-3.05
GFPT1	glutamine--fructose-6-phosphate transaminase 1	0.007	-3.05
STT3B	STT3, subunit of the oligosaccharyltransferase complex, homolog B (S. cerevisiae)	0.015	-3.05
LMO7	LIM domain 7	0.013	-3.05
ZNF750	zinc finger protein 750	0.043	-3.05
ARL5B	ADP-ribosylation factor-like 5B	0.003	-3.04
IPO7	importin 7	0.047	-3.04
COBLL1	COBL-like 1	0.012	-3.03
KIFC1	kinesin family member C1	0.024	-3.03
GALIG	lectin, galactoside-binding, soluble, 3	0.007	-3.02
STK39	serine threonine kinase 39	0.019	-3.02
KCNK1	potassium channel, subfamily K, member 1	0.018	-3.02
ABHD11	abhydrolase domain containing 11	0.004	-3.01
YME1L1	YME1-like 1 (S. cerevisiae)	0.007	-3.00
RUNX1	runt-related transcription factor 1	0.003	-3.00
OFD1	oral-facial-digital syndrome 1	0.007	-3.00
SEC11C	SEC11 homolog C (S. cerevisiae)	0.002	-3.00
SPTLC3	serine palmitoyltransferase, long chain base subunit 3	0.018	-3.00
CPM	carboxypeptidase M	0.004	-3.00
TMEM141	transmembrane protein 141	0.049	-2.99
LDLR	low density lipoprotein receptor	0.003	-2.98
SERPINB9	serpin peptidase inhibitor, clade B (ovalbumin), member 9	0.007	-2.98
DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	0.002	-2.98
ELL2	elongation factor, RNA polymerase II, 2	0.040	-2.98
PRC1	protein regulator of cytokinesis 1	0.024	-2.97
ARHGAP17	Rho GTPase activating protein 17	0.010	-2.97
FZD9	frizzled homolog 9 (Drosophila)	0.007	-2.97
STX3	syntaxin 3	0.014	-2.95
SLC39A6	solute carrier family 39 (zinc transporter), member 6	0.034	-2.95
FERMT2	fermitin family member 2	0.026	-2.95

SLC7A1	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	0.013	-2.95
ALG13	asparagine-linked glycosylation 13 homolog (S. cerevisiae)	0.015	-2.94
OPN3	opsin 3	0.048	-2.94
MAPK13	mitogen-activated protein kinase 13	0.014	-2.94
TES	testis derived transcript (3 LIM domains)	0.017	-2.94
KIAA0256	SECIS binding protein 2-like	0.009	-2.92
ZNF185	zinc finger protein 185 (LIM domain)	0.015	-2.92
ELF1	E74-like factor 1 (ets domain transcription factor)	0.009	-2.92
WDR43	WD repeat domain 43	0.043	-2.92
DDX52	DEAD (Asp-Glu-Ala-Asp) box polypeptide 52	0.005	-2.92
SBDSP	Shwachman-Bodian-Diamond syndrome pseudogene 1	0.005	-2.91
TP53BP2	tumor protein p53 binding protein, 2	0.022	-2.91
SRP19	signal recognition particle 19kDa	0.005	-2.91
FAM3C	family with sequence similarity 3, member C	0.031	-2.90
SNAP23	synaptosomal-associated protein, 23kDa	0.035	-2.89
GULP1	GULP, engulfment adaptor PTB domain containing 1	0.014	-2.89
FOXO1	forkhead box O1	0.005	-2.89
PIM1	pim-1 oncogene	0.002	-2.89
FAM152B	PPPDE peptidase domain containing 2	0.015	-2.88
CDR2	cerebellar degeneration-related protein 2, 62kDa	0.038	-2.88
ACSL5	acyl-CoA synthetase long-chain family member 5	0.044	-2.88
NDRG1	N-myc downstream regulated 1	0.000	-2.88
C9ORF150	chromosome 9 open reading frame 150	0.037	-2.87
PELI1	pellino homolog 1 (Drosophila)	0.040	-2.87
RAB21	RAB21, member RAS oncogene family	0.004	-2.87
EPS8	epidermal growth factor receptor pathway substrate 8	0.003	-2.87
GUSB	glucuronidase, beta	0.001	-2.87
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	0.014	-2.86
TNIP1	TNFAIP3 interacting protein 1	0.042	-2.86
VCL	vinculin	0.005	-2.86
FKBP4	FK506 binding protein 4, 59kDa	0.001	-2.86
MXD1	MAX dimerization protein 1	0.000	-2.85
PDLIM5	PDZ and LIM domain 5	0.018	-2.84
TNFAIP3	tumor necrosis factor, alpha-induced protein 3	0.000	-2.84
UGDH	UDP-glucose 6-dehydrogenase	0.002	-2.84
FAM100B	family with sequence similarity 100, member B	0.005	-2.83
IL1RL2	interleukin 1 receptor-like 2	0.005	-2.83
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1	0.016	-2.83
NOP5/NOP58	NOP58 ribonucleoprotein homolog (yeast)	0.007	-2.82
KRT80	keratin 80	0.025	-2.82
ERO1L	ERO1-like (S. cerevisiae)	0.006	-2.81
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)	0.011	-2.81
BTG3	BTG family, member 3	0.013	-2.81
ABHD5	abhydrolase domain containing 5	0.015	-2.80
EBP	emopamil binding protein (sterol isomerase)	0.004	-2.80
TBC1D15	TBC1 domain family, member 15	0.006	-2.80
UBAP1	ubiquitin associated protein 1	0.009	-2.80
CYB5A	cytochrome b5 type A (microsomal)	0.048	-2.80
C6ORF128	transmembrane protein 217	0.029	-2.79
RASSF1	Ras association (RalGDS/AF-6) domain family member 1	0.000	-2.79
MAP4K3	mitogen-activated protein kinase kinase kinase kinase 3	0.043	-2.79
AFF4	AF4/FMR2 family, member 4	0.023	-2.78
IL6ST	interleukin 6 signal transducer (gp130, oncostatin M receptor)	0.004	-2.78

MFHAS1	malignant fibrous histiocytoma amplified sequence 1	0.011	-2.77
UPP1	uridine phosphorylase 1	0.012	-2.77
LRRC1	leucine rich repeat containing 1	0.030	-2.77
SHRM	shroom family member 3	0.012	-2.76
BAZ1A	bromodomain adjacent to zinc finger domain, 1A	0.038	-2.76
FAM107B	family with sequence similarity 107, member B	0.008	-2.76
UFM1	ubiquitin-fold modifier 1	0.022	-2.76
DCI	enoyl-CoA delta isomerase 1	0.000	-2.76
LHFPL2	lipoma HMGIC fusion partner-like 2	0.043	-2.75
HM13	histocompatibility (minor) 13	0.002	-2.75
PYY	peptide YY	0.005	-2.75
ASAHL	N-acylethanolamine acid amidase	0.009	-2.75
CHD2	chromodomain helicase DNA binding protein 2	0.004	-2.75
BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like	0.004	-2.74
MREG	melanoregulin	0.011	-2.74
GMNN	geminin, DNA replication inhibitor	0.014	-2.74
TUBA1C	tubulin, alpha 1c	0.001	-2.74
PSMD14	proteasome (prosome, macropain) 26S subunit, non-ATPase, 14	0.022	-2.74
RAB5A	RAB5A, member RAS oncogene family	0.020	-2.73
MTE	metallothionein 1I (pseudogene)	0.028	-2.73
CRY1	cryptochrome 1 (photolyase-like)	0.044	-2.72
B4GALT4	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 4	0.003	-2.72
CLN8	ceroid-lipofuscinosis, neuronal 8 (epilepsy, progressive with mental retardation)	0.008	-2.72
ARHGAP21	Rho GTPase activating protein 21	0.013	-2.72
LAMP2	lysosomal-associated membrane protein 2	0.001	-2.72
DKFZP564O0523	chromosome 7 open reading frame 64	0.019	-2.72
CDV3	CDV3 homolog (mouse)	0.010	-2.71
MAP2K3	mitogen-activated protein kinase kinase 3	0.002	-2.71
PNRC2	proline-rich nuclear receptor coactivator 2	0.004	-2.71
PNPLA8	patatin-like phospholipase domain containing 8	0.017	-2.70
GNA13	guanine nucleotide binding protein (G protein), alpha 13	0.011	-2.70
USP9X	ubiquitin specific peptidase 9, X-linked	0.012	-2.70
MBP	myelin basic protein	0.040	-2.70
AK3L1	adenylate kinase 3	0.036	-2.70
RIOK3	RIO kinase 3 (yeast)	0.010	-2.69
SEC22B	SEC22 vesicle trafficking protein homolog B (S. cerevisiae) (gene/pseudogene)	0.001	-2.69
COPE	coatomer protein complex, subunit epsilon	0.000	-2.69
TMEM2	transmembrane protein 2	0.048	-2.69
B4GALT5	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 5	0.004	-2.68
JARID2	jumonji, AT rich interactive domain 2	0.049	-2.68
SLC30A7	solute carrier family 30 (zinc transporter), member 7	0.005	-2.68
CDS1	CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 1	0.022	-2.68
ARF4	ADP-ribosylation factor 4	0.001	-2.68
ACOT9	acyl-CoA thioesterase 9	0.021	-2.68
DDAH1	dimethylarginine dimethylaminohydrolase 1	0.003	-2.67
TAX1BP1	Tax1 (human T-cell leukemia virus type I) binding protein 1	0.002	-2.67
C12ORF28	chromosome 12 open reading frame 28	0.008	-2.67
MFAP5	microfibrillar associated protein 5	0.031	-2.67
SERTAD2	SERTA domain containing 2	0.022	-2.66

CAPZA2	capping protein (actin filament) muscle Z-line, alpha 2	0.002	-2.66
AGPS	alkylglycerone phosphate synthase	0.009	-2.66
AP1G1	adaptor-related protein complex 1, gamma 1 subunit	0.005	-2.66
GOLPH3	golgi phosphoprotein 3 (coat-protein)	0.029	-2.66
RAN	RAN, member RAS oncogene family	0.026	-2.66
NSDHL	NAD(P) dependent steroid dehydrogenase-like	0.010	-2.65
DSG2	desmoglein 2	0.011	-2.65
BRP44L	brain protein 44-like	0.005	-2.65
GLO1	glyoxalase I	0.011	-2.65
DLG5	discs, large homolog 5 (Drosophila)	0.005	-2.64
UBL3	ubiquitin-like 3	0.002	-2.63
MICB	MHC class I polypeptide-related sequence B	0.014	-2.63
ADIPOR2	adiponectin receptor 2	0.031	-2.63
ATP6V1A	ATPase, H+ transporting, lysosomal 70kDa, V1 subunit A	0.031	-2.62
HK2	hexokinase 2	0.013	-2.62
HSPB8	heat shock 22kDa protein 8	0.001	-2.62
TMEM49	vacuole membrane protein 1	0.001	-2.61
AMD1	adenosylmethionine decarboxylase 1	0.034	-2.61
MVD	mevalonate (diphospho) decarboxylase	0.033	-2.61
DHRS7	dehydrogenase/reductase (SDR family) member 7	0.007	-2.61
LRRC8A	leucine rich repeat containing 8 family, member A	0.039	-2.60
SELS	selenoprotein S	0.001	-2.60
NR1D2	nuclear receptor subfamily 1, group D, member 2	0.020	-2.60
LEPROT	leptin receptor overlapping transcript	0.030	-2.60
CPEB4	cytoplasmic polyadenylation element binding protein 4	0.020	-2.59
APOO	apolipoprotein O	0.010	-2.59
ATP6V0A1	ATPase, H+ transporting, lysosomal V0 subunit a1	0.005	-2.58
SPPL2A	signal peptide peptidase-like 2A	0.021	-2.58
CEACAM1	carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	0.011	-2.58
C17ORF91	chromosome 17 open reading frame 91	0.027	-2.58
PRPF40A	PRP40 pre-mRNA processing factor 40 homolog A (S. cerevisiae)	0.020	-2.58
RAB6IP1	DENN/MADD domain containing 5A	0.010	-2.57
SLC22A5	solute carrier family 22 (organic cation/carnitine transporter), member 5	0.018	-2.57
LRRC41	leucine rich repeat containing 41	0.006	-2.57
PTPN1	protein tyrosine phosphatase, non-receptor type 1	0.034	-2.56
MAL2	mal, T-cell differentiation protein 2 (gene/pseudogene)	0.026	-2.56
CICE	cell death-inducing DFFA-like effector c pseudogene	0.008	-2.56
TLCD1	TLC domain containing 1	0.019	-2.56
ARIH1	ariadne homolog, ubiquitin-conjugating enzyme E2 binding protein, 1 (Drosophila)	0.042	-2.56
TJP1	tight junction protein 1 (zona occludens 1)	0.007	-2.56
KPNA4	karyopherin alpha 4 (importin alpha 3)	0.014	-2.55
NUCB2	nucleobindin 2	0.010	-2.55
ACOT2	acyl-CoA thioesterase 2	0.007	-2.55
WWC1	WW and C2 domain containing 1	0.009	-2.55
ZBTB10	zinc finger and BTB domain containing 10	0.001	-2.55
CASP3	caspase 3, apoptosis-related cysteine peptidase	0.025	-2.55
RALA	v-ral simian leukemia viral oncogene homolog A (ras related)	0.029	-2.54
HERPUD1	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	0.048	-2.54

KDELR3	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3	0.033	-2.54
PLEKHA1	pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 1	0.031	-2.54
KCTD5	potassium channel tetramerisation domain containing 5	0.047	-2.53
PEA15	phosphoprotein enriched in astrocytes 15	0.013	-2.53
ELOVL5	ELOVL family member 5, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)	0.034	-2.53
DNM1L	dynamin 1-like	0.008	-2.53
S100A16	S100 calcium binding protein A16	0.039	-2.53
SGK	serum/glucocorticoid regulated kinase 1	0.003	-2.53
OGFOD1	2-oxoglutarate and iron-dependent oxygenase domain containing 1	0.005	-2.53
WDR1	WD repeat domain 1	0.008	-2.53
PTS	6-pyruvoyltetrahydropterin synthase	0.015	-2.52
CRB3	crumbs homolog 3 (Drosophila)	0.007	-2.52
RNF19A	ring finger protein 19A	0.008	-2.52
MAPK6	mitogen-activated protein kinase 6	0.019	-2.52
GABARAPL1	GABA(A) receptor-associated protein like 1	0.020	-2.52
NUP88	nucleoporin 88kDa	0.018	-2.52
TC2N	tandem C2 domains, nuclear	0.043	-2.51
KIAA1370	KIAA1370	0.021	-2.51
MTPN	myotrophin	0.007	-2.51
CACYBP	calcyclin binding protein	0.011	-2.51
MAP7	microtubule-associated protein 7	0.011	-2.51
HNRPH2	heterogeneous nuclear ribonucleoprotein H2 (H')	0.030	-2.51
IL8	interleukin 8	0.002	-2.51
BAIAP2L1	BAI1-associated protein 2-like 1	0.029	-2.50
LBR	lamin B receptor	0.015	-2.50
SLK	STE20-like kinase	0.023	-2.50
TMEM165	transmembrane protein 165	0.029	-2.50
HADHB	hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), beta subunit	0.012	-2.50
RCN1	reticulocalbin 1, EF-hand calcium binding domain	0.017	-2.49
KIF14	kinesin family member 14	0.048	-2.49
PELO	pelota homolog (Drosophila)	0.048	-2.49
RAB3IP	RAB3A interacting protein (rabin3)	0.011	-2.49
KRT19	keratin 19	0.016	-2.49
PRKAG2	protein kinase, AMP-activated, gamma 2 non-catalytic subunit	0.005	-2.49
SLC44A3	solute carrier family 44, member 3	0.020	-2.49
RRAGC	Ras-related GTP binding C	0.004	-2.49
RAPH1	Ras association (RalGDS/AF-6) and pleckstrin homology domains 1	0.021	-2.49
TMEM16K	anoctamin 10	0.007	-2.49
FGL1	fibrinogen-like 1	0.001	-2.48
RAB2A	RAB2A, member RAS oncogene family	0.009	-2.48
C1ORF122	chromosome 1 open reading frame 122	0.026	-2.48
SPRED2	sprouty-related, EVH1 domain containing 2	0.031	-2.48
NOLA3	NOP10 ribonucleoprotein homolog (yeast)	0.001	-2.48
PGK1	phosphoglycerate kinase 1	0.025	-2.48
MAEA	macrophage erythroblast attacher	0.032	-2.47
BIRC2	baculoviral IAP repeat containing 2	0.010	-2.47

SLC35A3	solute carrier family 35 (UDP-N-acetylglucosamine (UDP-GlcNAc) transporter), member A3	0.017	-2.47
ZNF622	zinc finger protein 622	0.015	-2.46
M6PRBP1	perilipin 3	0.003	-2.46
ARL8B	ADP-ribosylation factor-like 8B	0.009	-2.46
C1ORF108	akirin 1	0.043	-2.46
USP14	ubiquitin specific peptidase 14 (tRNA-guanine transglycosylase)	0.003	-2.46
COBL	cordon-bleu homolog (mouse)	0.045	-2.46
GNB5	guanine nucleotide binding protein (G protein), beta 5	0.019	-2.46
ANKRD57	ankyrin repeat domain 57	0.032	-2.45
HELZ	helicase with zinc finger	0.018	-2.45
TMEM170	transmembrane protein 170A	0.015	-2.45
DUSP6	dual specificity phosphatase 6	0.044	-2.45
MLL3	myeloid/lymphoid or mixed-lineage leukemia 3	0.020	-2.45
C14ORF129	chromosome 14 open reading frame 129	0.039	-2.44
P4HA1	prolyl 4-hydroxylase, alpha polypeptide I	0.004	-2.44
FASTKD5	FAST kinase domains 5	0.010	-2.44
SLC9A3R1	solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1	0.001	-2.44
CSNK1A1	casein kinase 1, alpha 1	0.016	-2.44
CLDN4	claudin 4	0.003	-2.44
GSR	glutathione reductase	0.009	-2.44
PRDX4	peroxiredoxin 4	0.001	-2.43
CTNNAL1	catenin (cadherin-associated protein), alpha-like 1	0.006	-2.43
MARCH7	membrane-associated ring finger (C3HC4) 7	0.019	-2.43
NET1	neuroepithelial cell transforming 1	0.003	-2.43
C6ORF115	chromosome 6 open reading frame 115	0.032	-2.42
VDAC1	voltage-dependent anion channel 1	0.005	-2.42
CLIP1	CAP-GLY domain containing linker protein 1	0.018	-2.42
FAM18B2	family with sequence similarity 18, member B2	0.020	-2.42
TCP1	t-complex 1	0.007	-2.42
SERP1	stress-associated endoplasmic reticulum protein 1	0.028	-2.42
ACSL4	acyl-CoA synthetase long-chain family member 4	0.020	-2.42
SQSTM1	sequestosome 1	0.002	-2.41
CYP2J2	cytochrome P450, family 2, subfamily J, polypeptide 2	0.047	-2.41
PPP2R1B	protein phosphatase 2, regulatory subunit A, beta	0.000	-2.41
SLC25A24	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 24	0.041	-2.41
UBE2D3	ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)	0.003	-2.41
UTP6	UTP6, small subunit (SSU) processome component, homolog (yeast)	0.020	-2.41
RAG1AP1	solute carrier family 50 (sugar transporter), member 1	0.004	-2.41
YIPF6	Yip1 domain family, member 6	0.009	-2.41
XPO6	exportin 6	0.011	-2.40
PSMD6	proteasome (prosome, macropain) 26S subunit, non-ATPase, 6	0.016	-2.40
GTPBP4	GTP binding protein 4	0.049	-2.40
MT1E	metallothionein 1E	0.015	-2.40
PDCL	phosducin-like	0.037	-2.40
RB1CC1	RB1-inducible coiled-coil 1	0.011	-2.40
ATP6V1H	ATPase, H+ transporting, lysosomal 50/57kDa, V1 subunit H	0.012	-2.39
SH3BGRL3	SH3 domain binding glutamic acid-rich protein like 3	0.025	-2.39
TM4SF1	transmembrane 4 L six family member 1	0.001	-2.39

MMP25	matrix metallopeptidase 25	0.045	-2.39
CTBP2	C-terminal binding protein 2	0.028	-2.39
PRRG4	proline rich Gla (G-carboxyglutamic acid) 4 (transmembrane)	0.005	-2.39
HIVEP2	human immunodeficiency virus type I enhancer binding protein 2	0.012	-2.38
DNAJC3	DnaJ (Hsp40) homolog, subfamily C, member 3	0.019	-2.38
CS	citrate synthase	0.011	-2.38
ARSG	arylsulfatase G	0.002	-2.38
DSP	desmoplakin	0.011	-2.38
C1QTNF6	C1q and tumor necrosis factor related protein 6	0.009	-2.38
SRP54	signal recognition particle 54kDa	0.006	-2.37
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	0.022	-2.37
PMM2	phosphomannomutase 2	0.001	-2.37
CDC5L	CDC5 cell division cycle 5-like (S. pombe)	0.015	-2.37
EIF3J	eukaryotic translation initiation factor 3, subunit J	0.035	-2.36
EHD4	EH-domain containing 4	0.014	-2.36
LATS2	LATS, large tumor suppressor, homolog 2 (Drosophila)	0.027	-2.36
NP	cardiotrophin 2, pseudogene	0.028	-2.36
PLAGL2	pleiomorphic adenoma gene-like 2	0.005	-2.36
SPAG4	sperm associated antigen 4	0.006	-2.36
PSCD1	cytohesin 1	0.030	-2.35
LONRF2	LON peptidase N-terminal domain and ring finger 2	0.025	-2.35
TANK	TRAF family member-associated NFKB activator	0.009	-2.35
CNKSR3	CNKSR family member 3	0.004	-2.35
PIK3C2A	phosphoinositide-3-kinase, class 2, alpha polypeptide	0.040	-2.35
TMEM136	transmembrane protein 136	0.032	-2.35
HBP1	HMG-box transcription factor 1	0.020	-2.35
ARRDC3	arrestin domain containing 3	0.008	-2.35
SH3GLB1	SH3-domain GRB2-like endophilin B1	0.024	-2.34
CHMP5	chromatin modifying protein 5	0.043	-2.34
MUSK	muscle, skeletal, receptor tyrosine kinase	0.002	-2.34
TBC1D10A	TBC1 domain family, member 10A	0.017	-2.34
FER1L3	myoferlin	0.028	-2.34
SRD5A3	steroid 5 alpha-reductase 3	0.010	-2.34
CKAP4	cytoskeleton-associated protein 4	0.022	-2.34
ABI1	abl-interactor 1	0.004	-2.34
RAD23B	RAD23 homolog B (S. cerevisiae)	0.049	-2.34
ZNF165	zinc finger protein 165	0.035	-2.34
TXNL2	glutaredoxin 3	0.029	-2.34
LOC653566	signal peptidase complex subunit 2 homolog pseudogene	0.045	-2.33
TFB2M	transcription factor B2, mitochondrial	0.044	-2.33
ARHGEF5	Rho guanine nucleotide exchange factor (GEF) 5	0.022	-2.33
WEE1	WEE1 homolog (S. pombe)	0.014	-2.33
OSTF1	osteoclast stimulating factor 1	0.006	-2.33
ARF1	ADP-ribosylation factor 1	0.001	-2.33
EIF5A	eukaryotic translation initiation factor 5A	0.020	-2.33
DCDC2	doublecortin domain containing 2	0.021	-2.33
C12ORF29	chromosome 12 open reading frame 29	0.035	-2.33
PITPNB	phosphatidylinositol transfer protein, beta	0.019	-2.33
EN2	engrailed homeobox 2	0.017	-2.33
PAF1	Paf1, RNA polymerase II associated factor, homolog (S. cerevisiae)	0.007	-2.32
ZW10	ZW10, kinetochore associated, homolog (Drosophila)	0.023	-2.32
ASGR1	asialoglycoprotein receptor 1	0.045	-2.32

SELK	selenoprotein K	0.038	-2.32
KPNA1	karyopherin alpha 1 (importin alpha 5)	0.012	-2.32
ATG16L1	ATG16 autophagy related 16-like 1 (S. cerevisiae)	0.009	-2.32
ENSA	endosulfine alpha	0.022	-2.32
UCHL3	ubiquitin carboxyl-terminal esterase L3 (ubiquitin thiolesterase)	0.048	-2.32
PTRH2	peptidyl-tRNA hydrolase 2	0.008	-2.31
ADM2	adrenomedullin 2	0.022	-2.31
CDCA4	cell division cycle associated 4	0.017	-2.31
ZDHHC9	zinc finger, DHHC-type containing 9	0.004	-2.31
ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	0.001	-2.31
COPG	coatomer protein complex, subunit gamma	0.018	-2.30
GTF3C6	general transcription factor IIIC, polypeptide 6, alpha 35kDa	0.035	-2.30
STT3A	STT3, subunit of the oligosaccharyltransferase complex, homolog A (S. cerevisiae)	0.030	-2.30
PAPOLA	poly(A) polymerase alpha	0.014	-2.30
STRN3	striatin, calmodulin binding protein 3	0.033	-2.29
BIN3	bridging integrator 3	0.002	-2.29
PLK4	polo-like kinase 4	0.035	-2.29
ZNF518B	zinc finger protein 518B	0.039	-2.29
SERINC3	serine incorporator 3	0.034	-2.29
MARCH3	membrane-associated ring finger (C3HC4) 3	0.036	-2.29
HERC4	hect domain and RLD 4	0.025	-2.29
SDCCAG1	nuclear export mediator factor	0.002	-2.29
ADK	adenosine kinase	0.021	-2.29
ATP2A2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	0.008	-2.29
CCNE1	cyclin E1	0.024	-2.28
P4HA2	prolyl 4-hydroxylase, alpha polypeptide II	0.022	-2.28
FAM59A	family with sequence similarity 59, member A	0.019	-2.28
CRK	v-crk sarcoma virus CT10 oncogene homolog (avian)	0.018	-2.28
C14ORF101	chromosome 14 open reading frame 101	0.022	-2.28
CA13	carbonic anhydrase XIII	0.034	-2.28
GCM1	glial cells missing homolog 1 (Drosophila)	0.012	-2.28
SFTPG	surfactant associated 2	0.026	-2.28
POPDC3	popeye domain containing 3	0.008	-2.27
ACTR2	ARP2 actin-related protein 2 homolog (yeast)	0.003	-2.27
PAWR	PRKC, apoptosis, WT1, regulator	0.045	-2.27
MCCC1	methylcrotonoyl-CoA carboxylase 1 (alpha)	0.044	-2.27
STBD1	starch binding domain 1	0.003	-2.27
PDIA6	protein disulfide isomerase family A, member 6	0.012	-2.27
MAP1LC3B	microtubule-associated protein 1 light chain 3 beta	0.006	-2.27
PPM1B	protein phosphatase, Mg2+/Mn2+ dependent, 1B	0.003	-2.27
DNAJB11	DnaJ (Hsp40) homolog, subfamily B, member 11	0.017	-2.27
LOC441511	mitofusin-1-like	0.036	-2.26
SENP5	SUMO1/sentrin specific peptidase 5	0.042	-2.26
WDSUB1	WD repeat, sterile alpha motif and U-box domain containing 1	0.007	-2.26
PATL1	protein associated with topoisomerase II homolog 1 (yeast)	0.047	-2.26
TMEM41B	transmembrane protein 41B	0.015	-2.26
C14ORF32	mitogen-activated protein kinase 1 interacting protein 1-like	0.028	-2.26
FAM46A	family with sequence similarity 46, member A	0.045	-2.26
C14ORF43	chromosome 14 open reading frame 43	0.009	-2.25
C20ORF111	chromosome 20 open reading frame 111	0.013	-2.25
KIF13B	kinesin family member 13B	0.042	-2.25

ECD	ecdysoneless homolog (Drosophila)	0.034	-2.25
MYO6	myosin VI	0.043	-2.25
IBTK	inhibitor of Bruton agammaglobulinemia tyrosine kinase	0.012	-2.25
ST7	suppression of tumorigenicity 7	0.014	-2.25
ERRFI1	ERBB receptor feedback inhibitor 1	0.022	-2.25
TBL1X	transducin (beta)-like 1X-linked	0.015	-2.25
MTP18	mitochondrial fission process 1	0.027	-2.25
CDK7	cyclin-dependent kinase 7	0.025	-2.25
ANXA2	annexin A2	0.002	-2.25
MPZL3	myelin protein zero-like 3	0.001	-2.24
MAPRE1	microtubule-associated protein, RP/EB family, member 1	0.011	-2.24
LONRF3	LON peptidase N-terminal domain and ring finger 3	0.038	-2.24
MRPS35	mitochondrial ribosomal protein S35	0.036	-2.24
BAT2D1	proline-rich coiled-coil 2C	0.028	-2.24
C19ORF53	chromosome 19 open reading frame 53	0.035	-2.24
ANKRD13A	ankyrin repeat domain 13A	0.030	-2.24
THOC4	THO complex 4	0.019	-2.24
SLC3A2	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	0.006	-2.24
C9ORF21	chromosome 9 open reading frame 21	0.022	-2.23
ANXA5	annexin A5	0.012	-2.23
ELOVL6	ELOVL family member 6, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)	0.047	-2.23
PPP2R5E	protein phosphatase 2, regulatory subunit B', epsilon isoform	0.017	-2.22
PHF5A	PHD finger protein 5A	0.007	-2.21
TPMT	thiopurine S-methyltransferase	0.012	-2.21
XPR1	xenotropic and polytropic retrovirus receptor 1	0.009	-2.21
RNASEH1	ribonuclease H1	0.037	-2.21
GATAD2A	GATA zinc finger domain containing 2A	0.007	-2.21
BAGE5	B melanoma antigen family, member 5	0.036	-2.21
SURF4	surfeit 4	0.022	-2.21
SNX30	sorting nexin family member 30	0.022	-2.20
RAB15	RAB15, member RAS onocogene family	0.038	-2.20
FICD	FIC domain containing	0.021	-2.20
TMEM85	transmembrane protein 85	0.007	-2.20
C19ORF10	chromosome 19 open reading frame 10	0.008	-2.20
SLC9A1	solute carrier family 9 (sodium/hydrogen exchanger), member 1	0.018	-2.19
IQGAP1	IQ motif containing GTPase activating protein 1	0.042	-2.19
ETNK1	ethanolamine kinase 1	0.021	-2.19
COQ10B	coenzyme Q10 homolog B (S. cerevisiae)	0.046	-2.19
TOR1AIP2	torsin A interacting protein 2	0.008	-2.19
ARMET	mesencephalic astrocyte-derived neurotrophic factor	0.013	-2.19
RPRC1	MAP7 domain containing 1	0.021	-2.19
STX5	syntaxin 5	0.011	-2.19
YWHAG	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide	0.020	-2.19
SBDS	Shwachman-Bodian-Diamond syndrome	0.027	-2.19
C10ORF46	chromosome 10 open reading frame 46	0.022	-2.18
C19ORF28	chromosome 19 open reading frame 28	0.042	-2.18
SPSB1	splA/ryanodine receptor domain and SOCS box containing 1	0.024	-2.18
HLA-E	major histocompatibility complex, class I, E	0.033	-2.18
GNL2	guanine nucleotide binding protein-like 2 (nucleolar)	0.010	-2.18
CLDN12	claudin 12	0.031	-2.18

RTKN	rhotekin	0.021	-2.18
CLDN3	claudin 3	0.001	-2.18
ANXA1	annexin A1	0.004	-2.18
EDG7	lysophosphatidic acid receptor 3	0.035	-2.17
KRTAP4-7	keratin associated protein 4-7	0.045	-2.17
SPCS3	signal peptidase complex subunit 3 homolog (S. cerevisiae)	0.015	-2.17
GMFB	glia maturation factor, beta	0.003	-2.17
LPAR1	lysophosphatidic acid receptor 1	0.040	-2.17
DDT	D-dopachrome tautomerase	0.015	-2.17
HECA	headcase homolog (Drosophila)	0.043	-2.17
ZNF410	zinc finger protein 410	0.021	-2.17
PSMC1	proteasome (prosome, macropain) 26S subunit, ATPase, 1	0.008	-2.17
RANGAP1	Ran GTPase activating protein 1	0.002	-2.17
ANXA11	annexin A11	0.049	-2.17
COQ7	coenzyme Q7 homolog, ubiquinone (yeast)	0.007	-2.17
IDI1	isopentenyl-diphosphate delta isomerase 1	0.012	-2.17
YES1	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1	0.035	-2.16
TACC1	transforming, acidic coiled-coil containing protein 1	0.012	-2.16
RALB	v-ral simian leukemia viral oncogene homolog B (ras related; GTP binding protein)	0.023	-2.16
BRAF	v-raf murine sarcoma viral oncogene homolog B1	0.009	-2.16
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	0.020	-2.16
TJP2	tight junction protein 2 (zona occludens 2)	0.026	-2.15
PPP2CA	protein phosphatase 2, catalytic subunit, alpha isozyme	0.008	-2.15
TUFT1	tuftelin 1	0.029	-2.15
MTR	5-methyltetrahydrofolate-homocysteine methyltransferase	0.011	-2.15
FUT4	fucosyltransferase 4 (alpha (1,3) fucosyltransferase, myeloid-specific)	0.004	-2.15
IFNE1	interferon, epsilon	0.008	-2.15
TAP1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	0.042	-2.15
PPHLN1	periphilin 1	0.005	-2.15
SOD2	superoxide dismutase 2, mitochondrial	0.042	-2.15
ACAT1	acetyl-CoA acetyltransferase 1	0.003	-2.14
GLIS3	GLIS family zinc finger 3	0.014	-2.14
TBC1D9	TBC1 domain family, member 9 (with GRAM domain)	0.047	-2.14
DPY19L1	dpy-19-like 1 (C. elegans)	0.045	-2.14
KPNA2	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	0.044	-2.14
SCYL2	SCY1-like 2 (S. cerevisiae)	0.022	-2.14
TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b	0.009	-2.14
CTH	cystathionase (cystathionine gamma-lyase)	0.039	-2.14
AKAP13	A kinase (PRKA) anchor protein 13	0.026	-2.13
HIST1H1C	histone cluster 1, H1c	0.012	-2.13
SEC61B	Sec61 beta subunit	0.006	-2.13
COPB2	coatomer protein complex, subunit beta 2 (beta prime)	0.014	-2.13
GMPPB	GDP-mannose pyrophosphorylase B	0.011	-2.13
TDG	thymine-DNA glycosylase	0.029	-2.13
EIF4E2	eukaryotic translation initiation factor 4E family member 2	0.007	-2.13
SNIP1	Smad nuclear interacting protein 1	0.020	-2.13
TALDO1	transaldolase 1	0.011	-2.13
PRPS2	phosphoribosyl pyrophosphate synthetase 2	0.028	-2.13
HNRNPC	heterogeneous nuclear ribonucleoprotein C (C1/C2)	0.041	-2.13
C1ORF77	chromatin target of PRMT1	0.005	-2.12

LDHA	lactate dehydrogenase A	0.026	-2.12
GCAT	glycine C-acetyltransferase	0.018	-2.12
MET	met proto-oncogene (hepatocyte growth factor receptor)	0.003	-2.12
MAP4K2	mitogen-activated protein kinase kinase kinase kinase 2	0.003	-2.12
ZBTB5	zinc finger and BTB domain containing 5	0.003	-2.12
CDKN2AIP	CDKN2A interacting protein	0.025	-2.12
BTBD6	BTB (POZ) domain containing 6	0.019	-2.12
KLF5	Kruppel-like factor 5 (intestinal)	0.031	-2.12
ALG9	asparagine-linked glycosylation 9, alpha-1,2-mannosyltransferase homolog (S. cerevisiae)	0.009	-2.12
WDR82	WD repeat domain 82	0.030	-2.12
BXDC1	ribosome production factor 2 homolog (S. cerevisiae)	0.018	-2.12
KIAA0251	pyridoxal-dependent decarboxylase domain containing 1	0.009	-2.11
FH	fumarate hydratase	0.033	-2.11
FKBP11	FK506 binding protein 11, 19 kDa	0.015	-2.11
ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	0.005	-2.11
SLC44A1	solute carrier family 44, member 1	0.045	-2.11
HIATL1	hippocampus abundant transcript-like 1	0.018	-2.11
ATP2C1	ATPase, Ca++ transporting, type 2C, member 1	0.036	-2.11
RBM4	RNA binding motif protein 4	0.027	-2.11
MAP4K5	mitogen-activated protein kinase kinase kinase kinase 5	0.016	-2.11
SCUBE2	signal peptide, CUB domain, EGF-like 2	0.039	-2.10
GJD3	gap junction protein, delta 3, 31.9kDa	0.022	-2.10
MGAT4A	mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-cetylglucosaminyltransferase, isozyme A	0.033	-2.10
MARK1	MAP/microtubule affinity-regulating kinase 1	0.015	-2.10
HIGD1A	HIG1 hypoxia inducible domain family, member 1A	0.029	-2.10
KRT18	keratin 18	0.042	-2.10
UAP1	UDP-N-acteylglucosamine pyrophosphorylase 1	0.008	-2.10
PPP2CB	protein phosphatase 2, catalytic subunit, beta isozyme	0.041	-2.10
GGT1	gamma-glutamyltransferase 1	0.030	-2.10
NDFIP1	Nedd4 family interacting protein 1	0.014	-2.09
TYW3	tRNA-yW synthesizing protein 3 homolog (S. cerevisiae)	0.034	-2.09
SETX	senataxin	0.049	-2.09
GSK3B	glycogen synthase kinase 3 beta	0.033	-2.09
TM9SF3	transmembrane 9 superfamily member 3	0.001	-2.09
SRP14P1	signal recognition particle 14kDa (homologous Alu RNA binding protein) pseudogene 1	0.038	-2.09
CLIC1	chloride intracellular channel 1	0.047	-2.09
TINF2	TERF1 (TRF1)-interacting nuclear factor 2	0.031	-2.09
CHCHD2	coiled-coil-helix-coiled-coil-helix domain containing 2	0.001	-2.08
C8ORF4	chromosome 8 open reading frame 4	0.016	-2.08
GTF2IP1	general transcription factor IIi, pseudogene 1	0.030	-2.08
RUSC1	RUN and SH3 domain containing 1	0.033	-2.08
KHSRP	KH-type splicing regulatory protein	0.017	-2.08
STX12	syntaxin 12	0.028	-2.08
ATP1B3	ATPase, Na+/K+ transporting, beta 3 polypeptide	0.012	-2.08
IKZF5	IKAROS family zinc finger 5 (Pegasus)	0.011	-2.08
ZSWIM6	zinc finger, SWIM-type containing 6	0.029	-2.08
TTLL12	tubulin tyrosine ligase-like family, member 12	0.032	-2.08
EIF1AX	eukaryotic translation initiation factor 1A, X-linked	0.033	-2.08
ZRANB1	zinc finger, RAN-binding domain containing 1	0.019	-2.08
FTH1	ferritin, heavy polypeptide 1	0.003	-2.07

PDGFRL	platelet-derived growth factor receptor-like	0.031	-2.07
RPN2	ribophorin II	0.014	-2.07
RABL3	RAB, member of RAS oncogene family-like 3	0.026	-2.07
STK3	serine/threonine kinase 3	0.003	-2.07
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	0.028	-2.07
RC3H2	ring finger and CCCH-type domains 2	0.020	-2.07
KIAA1754	inositol 1,4,5-triphosphate receptor interacting protein	0.014	-2.07
U2AF1	U2 small nuclear RNA auxiliary factor 1	0.007	-2.07
FNIP1	folliculin interacting protein 1	0.043	-2.07
NANS	N-acetylneuraminic acid synthase	0.025	-2.06
ARPC5L	actin related protein 2/3 complex, subunit 5-like	0.025	-2.06
FTHL3	ferritin, heavy polypeptide 1 pseudogene 3	0.013	-2.06
PRRC1	proline-rich coiled-coil 1	0.041	-2.06
ATP13A3	TPase type 13A3	0.034	-2.06
MGC40489	ypothetical LOC146880	0.033	-2.06
ETF1	eukaryotic translation termination factor 1	0.001	-2.06
NCOA4	nuclear receptor coactivator 4	0.003	-2.06
MYPN	myopalladin	0.044	-2.06
HSPA5	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	0.024	-2.05
MYNN	myoneurin	0.012	-2.05
ARCN1	archain 1	0.010	-2.05
JMJD1C	jumonji domain containing 1C	0.037	-2.05
GOLGB1	golgin B1	0.003	-2.05
LOC147804	ropomyosin 3 pseudogene	0.002	-2.05
FBXW11	F-box and WD repeat domain containing 11	0.039	-2.05
SIAH2	seven in absentia homolog 2 (Drosophila)	0.015	-2.05
NDRG4	NDRG family member 4	0.014	-2.05
CHMP2A	chromatin modifying protein 2A	0.008	-2.05
DYRK1A	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A	0.030	-2.04
SEPT10	septin 10	0.046	-2.04
HTATIP2	HIV-1 Tat interactive protein 2, 30kDa	0.039	-2.04
BCAP31	B-cell receptor-associated protein 31	0.001	-2.04
HERPUD2	HERPUD family member 2	0.021	-2.04
AADAC	arylacetamide deacetylase (esterase)	0.026	-2.04
GOLT1B	golgi transport 1B	0.025	-2.04
ETFDH	electron-transferring-flavoprotein dehydrogenase	0.031	-2.04
NAPSA	napsin A aspartic peptidase	0.038	-2.04
USO1	USO1 vesicle docking protein homolog (yeast)	0.003	-2.04
ILDR1	immunoglobulin-like domain containing receptor 1	0.024	-2.04
TCEB1	transcription elongation factor B (SIII), polypeptide 1 (15kDa, elongin C)	0.022	-2.04
SEC31A	SEC31 homolog A (S. cerevisiae)	0.009	-2.04
SMPD1	sphingomyelin phosphodiesterase 1, acid lysosomal	0.004	-2.03
HSPC111	NOP16 nucleolar protein homolog (yeast)	0.040	-2.03
TP53INP2	tumor protein p53 inducible nuclear protein 2	0.007	-2.03
SLC8A2	solute carrier family 8 (sodium/calcium exchanger), member 2	0.020	-2.03
TMIE	transmembrane inner ear	0.005	-2.03
SLC25A39	solute carrier family 25, member 39	0.009	-2.02
C16ORF44	kelch-like 36 (Drosophila)	0.019	-2.02
ZNF212	zinc finger protein 212	0.004	-2.02
TIMM23	translocase of inner mitochondrial membrane 23 homolog	0.012	-2.02

	(yeast)		
CNDP2	CNDP dipeptidase 2 (metallopeptidase M20 family)	0.042	-2.01
LOC729317	voltage-dependent anion channel 2 pseudogene	0.029	-2.01
PIPSL	PIP5K1A and PSMD4-like, pseudogene	0.032	-2.01
TRAM1	translocation associated membrane protein 1	0.033	-2.01
WDR44	WD repeat domain 44	0.047	-2.01
TNPO3	transportin 3	0.047	-2.01
SDHA	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	0.012	-2.01
PPM1A	protein phosphatase, Mg2+/Mn2+ dependent, 1A	0.028	-2.01
IRS2	insulin receptor substrate 2	0.023	-2.01
C9ORF61	family with sequence similarity 189, member A2	0.027	-2.00
PDIA3P	protein disulfide isomerase family A, member 3 pseudogene	0.017	-2.00
RNF103	ring finger protein 103	0.027	-2.00
OGT	O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase)	0.031	-2.00

Appendix 5 Focus genes for selected enriched gene ontology (GO) classifications of lowly expressed genes in Post-M *vs.* Pre-M

Enriched GO gene families	Fisher Exact test	Focus Genes
Biological processes		
Protein transport	2.36E-06	SEC31A, RAB9A, XPO6, CHMP5, AP1G1, PPARG, EIF5A, SRP19, RAB3IP, HOOK1, COPB2, RNF103, RAB21, ZW10, KIF13B, KDELR3, KDELR2, STX5, MYO6, STX3, NUP88, RAN, GOLT1B, TIMM23, BCAP31, SEC61B, KRT18, CD36, ATG4B, IPO7, ARCN1, RAB5A, USO1, RAB15, KPNA4, COPG, KPNA2, RAB11FIP1, KPNA1, COPE, SERP1, CHMP2A, YWHAZ, STX12, TAP2, TAP1, BCAP29, SEC22B, SNAP23, TRAM1, TNPO3, RAB2A, SRP54, RASEF, SRP14P1, SELS, YWHAG, ARF1, GSK3B, ARF4, SPTBN1, SNX30, ATG16L1
Protein localization	9.53E-06	SEC31A, RAB9A, XPO6, CHMP5, AP1G1, PPARG, EIF5A, SRP19, RAB3IP, HOOK1, COPB2, RNF103, RAB21, ZW10, KIF13B, KDELR3, KDELR2, STX5, MYO6, STX3, NUP88, RAN, GOLT1B, TIMM23, BCAP31, SEC61B, KRT18, CD36, ATG4B, IPO7, ARCN1, RAB5A, USO1, RAB15, BIN3, KPNA4, COPG, KPNA2, RAB11FIP1, COPE, KPNA1, SERP1, CHMP2A, YWHAZ, STX12, SH3GLB1, SQSTM1, TAP2, TAP1, BCAP29, SEC22B, SNAP23, TRAM1, TINF2, TNPO3, RAB2A, SRP54, RASEF, SRP14P1, ITGA2, SELS, YWHAG, ARF1, GSK3B, ARF4, SPTBN1, SNX30, ATG16L1
Sterol biosynthetic process	1.57E-05	EBP, CYB5R2, MVD, HMGCR, SQLE, PRKAG2, HMGCS1, IDI1, SC4MOL, NSDHL
Cofactor metabolic process	1.80E-05	MTHFD2L, TALDO1, HMGCR, CS, ACOT2, GSTT1, KMO, GGT1, COQ7, GCLM, SOD2, SDHA, ACOT9, MTHFD2, GSR, CTH, MCCC1, MTR, IDH1, VNN1, GLO1, QPRT, MGST1, FH
Glutathione metabolic process	2.02E-05	GSR, CTH, GSTT1, IDH1, GLO1, GGT1, GCLM, MGST1, SOD2
Vesicle targeting	3.10E-05	COPB2, STX5, ARF1, ARCN1, MAP4K2, SNAP23, COPG, COPE
Lipid biosynthetic process	4.49E-05	CYB5R2, HSD17B2, MVD, HMGCR, ABHD5, EDN1, PRKAG2, HMGCS1, ALG9, SC4MOL, NANS, AGPAT5, AGPS, ELOVL5, SH3GLB1, ETNK1, ELOVL6, ACSL3, NSDHL, PIGA, EBP, SPTLC3, PIK3C2A, UGCG, CDS1, PLAUR, ACSM3, C14ORF147, SQLE, SMPD1, IDI1, CLN8
Protein kinase cascade	4.73E-05	GNA13, IL6ST, EDN1, SNIP1, PRKAG2, MAP4K2, PRDX4, LPAR1, LATS2, RASGRP3, RB1CC1, PPP2CA, TICAM1, SPRED2, ADAM9, BCL10, MAP2K1, BRAF, MAP2K3, MET, STK3, MARK1, TANK, MAP4K3, RPS6KA5, TNFRSF10A, DUSP4, SPAG9, MAP4K5, TNFRSF10B, MAPK13, JAK1, NKIRAS1, DUSP6, SLC9A1
Negative regulation of cell death	1.47E-04	YWHAZ, MAEA, HTATIP2, FOXO1, BNIP3, NFKB1, GCLM, EDNRB, PEA15, CASP3, SH3GLB1, SQSTM1, RB1CC1, PPP2CB, VNN1, GLO1, HSPA5, BCL10, BRAF, SPHK1, PIM1, ANXA1, ANXA5, TAX1BP1, SELS, SOD2, SERPINB9, KRT18, GSK3B, BNIP3L, RIPK2, TNFAIP3, CLN8

Intracellular signalling cascade	2.14E-04	GNA13, RAB9A, IL6ST, RBM4, PRKAG2, EDN1, SNIP1, PRDX4, LPAR1, SDCBP2, IQGAP1, LATS2, EDNRB, CCNE1, TICAM1, RALB, SPRED2, RALA, DLG5, MLL3, ARL5B, DDAH1, RAB21, NET1, ADAM9, BCL10, MYO6, RABL3, BRAF, RXFP1, PIK3C2A, RAN, ARHGEF5, ADIPOR2, DCDC2, CDK7, STK3, TANK, MARK1, NRIP1, TNFRSF10A, MAP4K3, SPAG9, MAP4K5, CD36, TNFRSF10B, NCOA4, COL1A2, RAB5A, RAB15, ARL8B, SIAH2, NKIRAS1, MAP4K2, AKAP13, RTKN, GNG11, SFN, RRAGC, RASGRP3, SQSTM1, PPP2CA, RB1CC1, MT1H, RAB2A, SPSB1, MAP2K1, IL8, RASEF, MAP2K3, SPHK1, MET, RPS6KA5, DUSP4, ARF1, MAPK13, GSK3B, RASSF1, ARF4, JAK1, CRK, SLC9A1, DUSP6
Cholesterol biosynthetic process	5.11E-03	EBP, MVD, HMGCR, HMGCS1, IDI1, NSDHL
Positive regulation of cell migration	5.94E-03	SPAG9, IRS2, MAP2K1, IL6ST, PTP4A1, EDN1, SPHK1, F2RL1, ITGA2, IL6R, ADAM9
Wnt receptor signalling pathway	6.82E-02	FZD9, CSNK1A1, GSK3B, PPM1A, ZRANB1, TLE1, HBP1, LRRFIP2, SLC9A3R1, TBL1X, FBXW11
Regulation of cyclin-dependent protein kinase activity	3.16E-02	CASP3, GTPBP4, PIM1, CDK7, SFN, GADD45A, LATS2
Cell cycle	3.85E-02	KIFC1, MAEA, PRC1, USP9X, SENP5, WTAP, ITGB1, LATS2, CCNE1, SBDS, RAD21, RNF103, RB1CC1, HBP1, PSMD6, CABLES1, ZW10, MAP2K1, IL8, RAN, TP53BP2, GMNN, PIM1, ANXA1, LOC643668, NUSAP1, CDK7, CDC5L, SEPT10, WEE1, TACC1, PSMD14, KRT18, MAPK6, PSMC4, MAPK13, GSK3B, PTP4A1, RASSF1, PSMC1, CLIP1, PELO, BIN3, SIAH2, LOC652826, MAPRE1, KPNA2, GADD45A
Glucose metabolic process	3.96E-02	IRS2, LDHA, TALDO1, IL6ST, PRKAG2, UGDH, HK2, PGAM1, PGM3, PYGL, GSK3B, PGK1, SERP1
Cell division	3.97E-02	KIFC1, MAEA, PRC1, RAN, USP9X, NUSAP1, CDK7, SENP5, SEPT10, LATS2, TACC1, WEE1, CCNE1, RAD21, RNF103, ANXA11, PELO, BIN3, MAPRE1, CABLES1, ZW10
ATP biosynthetic process	4.36E-02	ATP6V1A, ATP6V1C2, ATP2A2, ATP1B3, ATP2C1, PRKAG2, ATP6V1H, ATP6V0A1, ATP13A3
Molecular functions		
Endoplasmic reticulum	3.76E-11	SEC31A, HM13, CYP2J2, RAB9A, TMEM189-UBE2V1, ARSG, HMGCR, ATL3, EIF5A, PDIA6, ALG9, SC4MOL, ELOVL5, P4HA2, DNAJB11, P4HA1, PLOD2, SRD5A3, LOC646817, RPN2, ELOVL6, DNAJC3, KCNG3, RAB21, ZW10, PIGA, KDELR3, TOR1AIP2, KDELR2, STX5, GOLT1B, SPTLC3, UGCG, CYB5A, TMEM189, CDS1, BCAP31, AADAC, PNPLA8, C14ORF147, NPC1, SEC61B, SQLE, NUCB2, SURF4, CLN8, SERP1, HSD17B2, ABI1, STT3B, STT3A, TAP2, ZDHHC9, TAP1, BCAP29, SEC22B, SLC39A6, ERO1L, HSPA5, ACSL4, ACSL3, TRAM1, HLA-DOB, EHD4, NSDHL, ACSL5, RAB2A, EBP, GABARAPL1, HERPUD1, DNM1L, YIPF6, SEC11C, CKAP4, SELK, LOC653566, SELS, GJB1, TMEM49, C3ORF52, DNAJB9, ATP2A2, PTP4A1, BNIP3L, SPCS3, PTPN1, MGST1, RCN1, ALG13

Endomembrane system	2.77E-09	SEC31A, RAB9A, HTATIP2, LDLR, XPO6, HMGCR, AP1G1, BNIP3, EIF5A, RANGAP1, WTAP, ALG9, SLC35A3, SC4MOL, EDNRB, COPB2, DAB2, RC3H2, RPN2, ELOVL6, LBR, RAB21, PIGA, STX5, MYO6, NUP88, RAN, SPTLC3, CLIC1, CDS1, BCAP31, AADAC, C14ORF147, NPC1, SEC61B, CD36, SQLE, ATP2C1, IPO7, NUCB2, ARCN1, USO1, RAB5A, KPNA4, COPG, KPNA2, RAB11FIP1, KPNA1, COPE, HSD17B2, MAL2, MAP4K2, STT3B, STT3A, CACYBP, ERO1L, B3GNT2, HSPA5, TRAM1, NSDHL, HERPUD1, SEC11C, LOC653566, SELS, GJB1, LAMP2, ARF1, ATP2A2, BNIP3L, ANXA11, SPCS3, GOLGB1
Cytosol	8.38E-09	STIL, LDHA, AP1G1, CHMP5, PDLIM5, PGAM1, FOXO1, EIF5A, ILDR1, VCL, NANS, EIF1AX, OGT, NET1, BCL10, MYO6, RAN, BCAP31, USO1, RIPK2, CHMP2A, TALDO1, MVD, HMGCS1, AKAP13, KMO, GCLM, FTH1, LOC647859, IDH1, EIF3J, AMD1, MAP2K1, SPHK1, GARS, PLK4, ARF1, PSMC4, MTR, PSMC1, SPTBN1, QPRT, PTPN1, WDR1, LOC652826, IDI1, PRKAG2, PPARG, NFKB1, RAB3IP, CKB, OFD1, GSR, CCNE1, COPB2, CASP3, AGPAT5, RNF103, TICAM1, LOC646817, PSMD6, CABLES1, PIK3C2A, NUP88, LOC643668, ATP6V1H, HERC4, CLIC1, SERPINB9, ATP6V1A, ADK, SERPINB8, ARCN1, NUCB2, TXNRD1, MAPRE1, WDR44, COPG, SMS, COPE, KPNA1, ABHD5, UPP1, UGDH, HK2, ABI1, HPRT1, FTHL3, SQSTM1, PPP2CA, TAP2, PPP3CC, HSPA5, YES1, GLRX, CSNK1A1, TCP1, DNM1L, TPMT, BIRC2, YWHAG, PSMD14, GSK3B, GOLPH3, TCEB1, CRK
Organelle membrane	2.71E-08	SEC31A, RAB9A, LDLR, AP1G1, HMGCR, GCAT, BNIP3, WTAP, ALG9, SLC35A3, SC4MOL, HOOK1, EDNRB, COPB2, DAB2, AGPS, RNF103, MAP1LC3B, SLC25A24, MCCC1, RPN2, ELOVL6, LBR, RAB21, PIGA, STX5, MYO6, SPTLC3, ATP6V1H, CYB5A, SLC3A1, CDS1, TIMM23, BCAP31, AADAC, PNPLA8, C14ORF147, SEC61B, CD36, SQLE, ATP2C1, SCYL2, NUCB2, ARCN1, USO1, RAB5A, SLC25A39, ARL8B, WDR44, COPG, RAB11FIP1, COPE, HSD17B2, MAP4K2, HK2, KMO, ACAT1, DCI, HADHB, STT3B, STT3A, SH3GLB1, CKMT2, ETFDH, B3GNT2, ERO1L, HSPA5, ACSL4, ACSL3, TRAM1, NSDHL, ACSL5, HERPUD1, DNM1L, SEC11C, LOC653566, SELS, GJB1, SOD2, VDAC1, SDHA, LAMP2, ATP2A2, ARF1, SPCS3, BRP44L, MGST1, GOLGB1
Endoplasmic reticulum part	3.66E-06	RAB9A, HSD17B2, HMGCR, PDIA6, ALG9, SC4MOL, STT3B, STT3A, DNAJB11, P4HA2, P4HA1, TAP2, TAP1, HSPA5, RPN2, ELOVL6, ERO1L, HLA-DOB, TRAM1, NSDHL, PIGA, HERPUD1, SEC11C, SPTLC3, LOC653566, CDS1, SELS, GJB1, BCAP31, AADAC, C14ORF147, SEC61B, ATP2A2, SQLE, SPCS3, RCN1
Nuclear envelope-endoplasmic reticulum network	1.99E-05	RAB9A, HSD17B2, HMGCR, EIF5A, ALG9, SC4MOL, STT3B, STT3A, HSPA5, ELOVL6, ERO1L, RPN2, TRAM1, NSDHL, PIGA, HERPUD1, SEC11C, SPTLC3, LOC653566, CDS1, SELS, GJB1, BCAP31, AADAC, C14ORF147, SEC61B, ATP2A2, SQLE, NUCB2, SPCS3

Nucleotide binding	1.84E-04	GNA13, KIFC1, RAB9A, HMGCR, RBM4, ITPKC, PRKX, SETX, ACTG2, AGPS, U2AF1, RALB, RALA, STK39, CRY1, GNL2, RAB21, KIF13B, GTPBP4, MYO6, BRAF, RAN, PIM1, WEE1, MARK1, STK3, MAP4K3, PNPLA8, MAP4K5, MAPK6, RIOK3, ATP2C1, SCYL2, RIPK2, RAB15, YME1L1, ARL8B, FASTKD5, PRPS2, PCCA, MVD, MAP4K2, KMO, HADHB, RRAGC, GMPPB, MUSK, UBE2D3, IDH1, HNRNPC, ERO1L, RUNX1, HLA-DOB, RHOBTB3, SRP54, MAP2K1, RASEF, MAP2K3, CNKSR3, SPHK1, MET, GARS, ATP13A3, SEPT10, PLK4, PSMC4, ATP2A2, ARF1, DYRK1A, PSMC1, ARF4, LOC652826, DDX52, STEAP4, CPEB4, ATL3, PRKAG2, HELZ, LATS2, CKB, ACTR2, MCCC2, GSR, SLK, MCCC1, SLC22A5, ARL5B, TUBA1C, KIF14, CTBP2, RABL3, PIK3C2A, ARHGEF5, LOC643668, CDK7, UBE2H, ATP6V1A, SQLE, ADK, RAB5A, THOC4, TXNRD1, NKIRAS1, FKBP4, HK2, UGDH, RTKN, AK3L1, STK40, CKMT2, TAP2, TAP1, ETFDH, CHD2, ETNK1, HSPA5, ACSL4, PAPSS1, YES1, ACSL3, EHD4, ACSL5, RAB2A, CSNK1A1, TCP1, DNM1L, SDHA, ACSM3, RPS6KA5, PAPOLA, PYGL, MAPK13, GSK3B, JAK1, PGK1
ER-Golgi intermediate compartment	1.83E-03	C19ORF10, NUCB2, SURF4, SEC22B, PDIA6, HSPA5, CLN8, GOLGB1
Protein binding, bridging	2.76E-03	EPS8, TP53BP2, FGB, FKBP4, RUSC1, RBM4, COL1A2, GATAD2A, ANXA1, DSP, HSPA5, CRK
ATP binding	5.79E-03	KIFC1, PRKAG2, HELZ, ITPKC, PRKX, LATS2, SETX, CKB, ACTG2, ACTR2, MCCC2, SLK, MCCC1, STK39, SLC22A5, KIF13B, KIF14, MYO6, BRAF, PIK3C2A, PIM1, LOC643668, UBE2H, CDK7, STK3, WEE1, MARK1, MAP4K3, PNPLA8, ATP6V1A, MAP4K5, MAPK6, ATP2C1, SCYL2, RIOK3, ADK, RIPK2, YME1L1, FASTKD5, PRPS2, PCCA, MVD, FKBP4, MAP4K2, HK2, AK3L1, UBE2D3, MUSK, STK40, TAP2, CKMT2, TAP1, CHD2, ETNK1, HSPA5, ACSL4, PAPSS1, YES1, RUNX1, HLA-DOB, ACSL3, EHD4, RHOBTB3, ACSL5, CSNK1A1, TCP1, MAP2K1, MAP2K3, SPHK1, CNKSR3, MET, GARS, ATP13A3, ACSM3, RPS6KA5, PAPOLA, PLK4, ATP2A2, PSMC4, PYGL, MAPK13, GSK3B, DYRK1A, PSMC1, JAK1, LOC652826, PGK1, DDX52
Kinase binding	1.28E-02	IBTK, BCL10, IRS2, MAP2K1, BRAF, PDLIM5, PRKAG2, PGAM1, FTH1, SPAG9, YWHAG, RASGRP3, SQSTM1, FTHL3, GSK3B, ADAM9, KIF13B
Fatty acid binding	2.46E-02	ALDH6A1, STX3, SH3GLB1, PPARG, PECI, HADHB
Steroid hormone receptor binding	2.46E-02	CCNE1, NCOA4, RAN, FKBP4, CDK7, NRIP1
Enzyme binding	3.77E-02	C10ORF46, PDLIM5, PRKAG2, PGAM1, RTKN, PAWR, GCLM, FTH1, RAB3IP, IQGAP1, RASGRP3, FTHL3, SQSTM1, RHOBTB3, ADAM9, KIF13B, IBTK, BCL10, IRS2, MAP2K1, BRAF, STRN3, IL6R, SELS, PLAUR, ANXA2, NRIP1, SPAG9, YWHAG, IPO7, GSK3B, ATP6V0A1, WDR44, KPNA2
Cellular components		
Extracellular region	2.88E-04	SCPEP1, FGF18, LTBP3, FAM20A, FGF9, LY86, FAM20C, SORL1, MMP7, NOTUM, HP, C1QC, CFI, VWA1, LTB, MATN2, WNT10A, PLXNB1, CCL4L1, MFGE8, MMP11, C1QB, PLA2G7, MFAP2, ADAMTS1, SCGB3A1, WFDC2, FMOD, TNFRSF25, C1QTNF5, COL9A2, FCN1, MSLN, FBN2, THBS4, RNASE1, PLEK, OLR1, CFB, TNFSF9,

		LGALS9, LCN2, TNFSF10, ACPL2, NOTCH4, C1RL, CMTM7, FCGBP, CD14, DMBT1
Microtubule cytoskeleton	3.49E-03	KIFC1, MAEA, PRC1, LATS2, RAB3IP, HOOK1, OFD1, CCNE1, PEA15, SBDS, MAP1LC3B, PPP2CA, PPP2CB, TUBA1C, ZW10, KIF13B, KIF14, GABARAPL1, TCP1, MAP2K1, RNF19A, DYNLT3, NUSAP1, TACC1, MARK1, PLK4, KRT18, ATG4B, PTP4A1, RASSF1, SPAG4, ANXA11, CLIP1, MAP7, ARL8B, MAPRE1, TNFAIP3, WDR44, TBL1X
Mitochondrial outer membrane	5.90E-03	DNM1L, SH3GLB1, HK2, BNIP3, CYB5A, KMO, ACSL4, ACSL3, MGST1, ACSL5, VDAC1
Cytoplasmic membrane-bounded vesicle	6.21E-03	GNA13, YWHAZ, SEC31A, LDLR, ATP1B3, AP1G1, PDIA6, ITGB1, COPB2, DAB2, STX12, FGB, SEC22B, SLC30A2, HSPA5, SNAP23, RAB21, SLC30A7, RAB2A, MYO6, STX3, PIK3C2A, RAN, GARS, SLC3A2, ANXA2, SPAG9, LAMP2, CD36, SCYL2, ARCN1, ANXA11, SMPD1, RAB5A, ATP6V0A1, COPG, RAB11FIP1, COPE
Cell-cell junction	9.39E-03	STX3, GJD3, CLDN4, CLDN3, CNKSR3, LMO7, CRB3, ARHGAP17, CLDN12, GJB1, VCL, TJP1, DSG2, LOC647859, DSP, DLG5, TJP2
Apicolateral plasma membrane	1.38E-02	TJP1, DSG2, CLDN4, LOC647859, CLDN3, CNKSR3, DSP, CRB3, ARHGAP17, CLDN12, TJP2

This table was generated by DAVID functional classification tool from the low expression gene list (Appendix 4).