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Characterization of Human Endometrial Glandular Epithelium *In Vitro and In Vivo*

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A thesis submitted to the University of Warwick for the
degree of Doctor of Philosophy.

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Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree.

The work presented (including data generated and data analysis) was carried out by the author except in the cases outlined below:

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Abstract

Endometrial glands provide histiotrophic support for the developing conceptus prior to the onset of the haemochorial placenta. Several lines of evidence suggest that glands also play an important role in endometrial receptivity, decidualization of the stromal compartment, and in maternal immune tolerance during pregnancy. However, glandular epithelial cells isolated during the luteal phase become acutely arrested *in vitro*, precluding in-depth analysis of this cellular compartment of the human endometrium. In this thesis, I tested various approaches to overcome the senescence-associated cell cycle block of primary human endometrial epithelial cells (HEECs). I demonstrate that conditional reprogramming of HEECs, using conditioned medium of irradiated fibroblast and a Rho kinase inhibitor (Y-27632), partially reverses the senescent phenotype and enables expansion of primary HEEC cultures. However, the responsiveness of reprogrammed HEECs to embryo-derived signals and hormonal cues remained highly variable. To overcome this hurdle, I used a novel 3D culture system that enabled formation of glandular structures from clonal HEECs seeded in Matrigel and cultured in modified adult stem cell medium. Treatment of glandular organoids with cyclic AMP and steroid hormones induced the expression of *PAEP*, a glandular differentiation marker. Organoid-forming efficiency experiments revealed that missed miscarriage, characterized by early-onset foetal growth retardation, is associated with glandular progenitor cell deficiency. To validate this observation, I used laser capture microdissection coupled to RNA-sequencing to compare mid-luteal glandular gene expression between missed miscarriage cases and control subjects. Gene ontology analysis of differentially expressed genes revealed that miscarriage may be caused by bioenergetics defects in the glands, exemplified by altered expression of mitochondrial-related genes. Taken together, the ability to grow and differentiate endometrial glands from isolated clonal HEECs provides a powerful new tool to study the mechanisms underpinning reproductive failure.

List of Abbreviations

2D	two dimensions
3D	three dimension
3T3 SAF	3T3 swiss albino embryo fibroblasts
11βHSD11	11 β hydroxysteroid dehydrogenase-1
μg	microgram
μl	microliter
μM	micromol/l
μm	micron or micrometer
AC	adenylyl cyclase
ADAM17	ADAM metallopeptidase domain 17
ALCAM	activated leukocyte cell adhesion molecule
APS	Ammonium persulfate
AQP	aquaporin
ATP	adenosine triphosphate
a.u.	arbitrary units
BAX	BCL2 associated X, apoptosis regulator
BCL2	B-cell lymphoma 2
bFGF	basic fibroblast growth factor
BMI	body mass index
BMP	bone morphogenetic protein
BSA	bovine serum albumin
C	control group
Ca²⁺	calcium ion
cAMP	8-bromo-cyclic adenosine monophosphate
CARM1	coactivator associated arginine methyltransferase 1
CBP	CREB binding protein
CCD	charge-coupled device
CD44	CD44 molecule (indian blood group)
CDH1	e-cadherin

CDK4	cyclin-dependent kinase 4
cDNA	complementary deoxyribonucleic acid
C/EBPβ	CCAAT/enhancer-binding protein β
CK18	cytokeratin 18
CM_i	conditioned medium from irradiated 3T3 SAF
CO₂	carbon dioxide
COX₂	cyclooxygenase 2
CRB	crumbs proteins
CRC	conditional reprogramming of cells
CRE	cAMP response element
CREB	cAMP response element binding protein
CREM	cAMP response element modulator
CRH	corticotropin releasing hormone
CRL	crown-rump length
CSF1	colony stimulating factor 1
CTNNB1	β -catenin
D0	conditioned media from un-decidualized stromal cells
D4	conditioned media from stromal cells decidualized for 4 days
D10	conditioned media from stromal cells decidualized for 10 days
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cells
DCC	dextran coated charcoal
DCE	developmentally competent embryos
DEF	defensins
DEG	differentially expressed gene
DHT	dihydrotestosterone
DIE	developmentally impaired embryos
DLL1	delta like canonical notch ligand 1
DLL4	delta-like ligand 4
DMEM/F12	Dulbeco's modified Eagle medium/F12 nutrient mix (Ham)
DMSO	dimethyl sulfoxide

DNA	deoxyribonucleic acid
DPP4	dipeptidyl peptidase 4
Dsh	dishevelled proteins
DTT	dithiothreitol
E2	oestradiol
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ENaC	epithelial sodium channel
EP2	prostaglandin E ₂ receptor 2
EP4	prostaglandin E ₂ receptor 4
ER	oestrogen receptor
ERBB4	receptor tyrosine-protein kinase erbB-4
ERK	extracellular signal-regulated kinase
ES	early secretory endometrium
EVT	extravillous trophoblast
FAS	Fas cell surface death receptor
FASL	FAS ligand
FDP	fibrin degradation products
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FKBP52	FK506-binding protein 4
FOXO1	forkhead box protein O1
FSH	follicle-stimulating hormone
FU	fluorescence intensity unit
FZD	frizzled receptors
GADD45α	growth arrest and DNA damage 45 α
GAG	glycosaminoglycans
gDNA	genomic deoxyribonucleic acid

GE	glandular epithelium
GEO	gene expression omnibus
GM-CSF	granulocyte macrophage colony-stimulating factor
GO	gene ontology
GPCR	G-protein-coupled receptor
GPX	glutathione peroxidase
GS	gestational sac
GSTM1	glutathione S-transferase M1
GSTT1	glutathione S-transferase T1
H&E	haematoxylin and eosin
H3K9me3	histone 3 tri-methyl K9
HAND2	heart and neural crest derivatives expressed 2
HB-EGF	heparin-binding EGF-like growth factor
hCG	human chorionic gonadotropin
HDM2	human double minute 2
HECA 452	cutaneous lymphocyte-associated antigen
HEECs	human endometrial epithelial cells
HESCs	human endometrial stromal cells
HGF	hepatocyte growth factor
HIF1α	hypoxia induced factor 1
HMGB2	high mobility group box 2 protein
HOXA	homeobox A
hPL	human placental lactogen
ICER	inducible cAMP early repressor
ICM	inner cell mass
IDO	indoleamine 2,3-dioxygenase
IFNγ	interferon γ
IGF	insulin-like growth factor
IHC	immunohistochemistry
IHH	indian hedgehog
IL	interleukin

iPSC	induced pluripotent stem cells
IU	international unit
IVS	intervillous space
J/m²	Joules/square metres
JAK	Janus kinase
kDa	kilodalton
KLF15	Krüppel-like factor 15
LDS	lithium dodecyl sulfate
LE	luminal epithelium
LGR	leucine-rich repeat-containing G-protein coupled receptor
LH	luteinizing hormone
LIF	leukaemia inhibitory factor
LMD	laser microdissection
LPR1	low-density lipoprotein receptor-related protein-1
LS	late secretory endometrium
M	mol/l
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein 1
Mcm2	DNA replication licensing factor MCM2
MECA 79	high endothelial venule marker
mg	milligram
ml	millilitre
mM	milimol/l
mm	millimetre
MMP	matrix metalloprotease
MPA	medroxyprogesterone acetate
mRNA	messenger ribonucleic acid
MS	mid-secretory endometrium
MSX	Msh homeobox
MUC1	mucin 1
MYC	vian myelocytomatosis viral oncogene homolog

<i>n</i>	sample size
NANOG	Nanog homeobox
NCOR	nuclear receptor co-repressor 1
NCS	nucleolar channel system
NF-κB	nuclear factor kappa b
ng	nanogram
NGF	nerve growth factor
nM	nanomol/l
nm	nanometres
OCT4	octamer-binding transcription factor 4
OFE	organoid forming efficiency assay
<i>P</i>	p-value
P0	passage zero
P1	passage one
P4	progesterone
P5/6	proprotein convertase 5/6
P16	cyclin-dependent kinase inhibitor 2A, multiple tumor suppressor 1
P21	cyclin-dependent kinase inhibitor 1
P53	tumor suppressor p53
P63	tumor suppressor p63
P_o	open time probability
PAF	platelet-activated factor
PAI-1	plasminogen activator inhibitor 1
PAPPA	pregnancy-associated plasma protein A
PAR	protease activated receptor
PBS	phosphate buffered saline
pCAF	p300/CBP associated factor
PCOS	polycystic ovarian syndrome
PCR	polymerase chain reaction
PDE	phosphodiesterase
PDGF	platelet derived growth factor

PG	prostaglandin
pg	pictogram
PGD	preimplantation genetic diagnosis
PGE₂	prostaglandin E ₂
PGF₂α	prostaglandin F ₂ α
PI3K/AKT	phosphoinositide 3-kinase/protein kinase B
PIF	preimplantation factor
PIGF	placental growth factor
PKA	cAMP-dependent protein kinase
PLA2	phospholipase A2
PMSF	phenylmethylsulfonyl fluoride
PR	progesterone receptor
PRL	prolactin
PRLR	prolactin receptor
PUGKO	progesterin uterine gland knock-out
PVDF	polyvinylidene fluoride
R	conditionally reprogrammed human endometrial epithelial cells
RANTES	regulated on activation, normal T cell expressed and secreted
Rb	retinoblastoma protein
Rho	RAS homolog
RI	rho-associated protein kinase (ROCK) inhibitor
RIN	RNA integrity number
RIPA	radioimmunoprecipitation assay
RMM	recurrent missed miscarriage
RNA	ribonucleic acid
ROCK	rho-associated protein kinase
ROS	reactive oxygen species
RPL	recurrent pregnancy loss
RTCA	real-time cell analysis
RT-qPCR	real-time quantitative polymerase chain reaction

RW	conditionally reprogrammed endometrial epithelial cells, followed by reprogramming withdrawal
S	monoculture of HESCs
S1P	sphingosine 1-phosphate
S/E	co-culture of HESCs and HEECs
SA-β-gal	senescence-associated beta-galactosidase
SCNT	somatic cell nuclear transfer
SCRIB	scribble protein
SD	standard deviation
SDS	sodium dodecyl sulphate
sFLT1	soluble fms-like tyrosine kinase 1
SGK1	serum and glucocorticoid-regulated kinase 1
SLPI	secretory leukocyte protease inhibitor
SMRT	nuclear receptor co-repressor 2
SOD	superoxide dismutase
SOX	SRY-box
SPINK	serine peptidase inhibitor, Kazal
SPP1	osteopontin
SRC2	nuclear receptor coactivator 2
STAT	signal transducer and activator of transcription
SUMO	small ubiquitin-related modifier
TBE	tris/borate/EDTA
TBS	tris buffered saline
TBS-T	tris buffered saline with Tween 20
TCF/LEF	T-cell factor/lymphoid-enhancing factor
TE	tris/EDTA
TEMED	N, N, N',N'-tetramethylethylenediamine
TF	tissue factor
TGFβ	transforming growth factor beta
TNFα	tumour necrosis factor α
TIMP	tissue inhibitor of matrix metalloproteases

tPA	tissue plasminogen activator
Treg	regulatory T cells
TUNEL	deoxynucleotidyl transferase dUTP Nick-end labeling
UGKO	uterine gland knock-out
ULF	uterine luminal fluid
uNK	uterine natural killer cell
uPA	urokinase plasminogen activator
UTMP	uterine milk protein
UVC	ultraviolet light C
VEGF	vascular endothelial growth factor
WAP	whey acidic proteins
WNT	wingless-type MMTV integration site family member
WOI	window of implantation

Genes

<i>ALKBH7</i>	AlkB homolog 7
<i>APOPT1</i>	apoptogenic 1, mitochondrial
<i>ATP5E</i> ATP	synthase, H ⁺ transporting, mitochondrial Fo complex epsilon subunit
<i>ATP5G3</i>	ATP synthase, H ⁺ transporting, mitochondrial Fo complex subunit C3 (subunit 9)
<i>ATP5I</i>	ATP synthase, H ⁺ transporting, mitochondrial Fo complex subunit E
<i>ATP5J2</i>	ATP synthase, H ⁺ transporting, mitochondrial Fo complex subunit F2
<i>ATP5L</i>	ATP synthase, H ⁺ transporting, mitochondrial Fo complex subunit G
<i>COA3</i>	cytochrome C oxidase assembly factor 3
<i>COX5A</i>	cytochrome C oxidase subunit 5A
<i>COX5B</i>	cytochrome C oxidase subunit 5B
<i>COX6A1</i>	cytochrome C oxidase subunit 6A1
<i>COX7A2</i>	cytochrome C oxidase subunit 7A2

COX7B	cytochrome C oxidase subunit 7B
COX8A	cytochrome C oxidase subunit 8A
ESR1	oestrogen receptor alpha
F2RL1	F2R like trypsin receptor
FAM89A	family with sequence similarity 89 member A
GRIM19	gene associated with retinoid-interferon mortality 19
HAND2	heart and neural crest derivatives expressed 2
L19	ribosomal protein 19
LHCGR	luteinizing Hormone/chorionic gonadotropin receptor
MFAP4	microfibrillar associated protein 4
MT-ATP6	mitochondrially encoded ATP synthase 6
MTCH1	mitochondrial carrier 1
MT-CO2	mitochondrially encoded cytochrome C oxidase III
MT-CO3	mitochondrially encoded cytochrome C oxidase II
MT-ND2	mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 2
MT-ND3	mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 3
MT-ND4	mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 4
NID2	nidogen 2
OSER1	oxidative stress responsive serine rich 1
PAEP	progesterone associated endometrial protein
PGR	progesterone receptor
PRDX5	peroxiredoxin 5
PRL	prolactin
PROK1	prokineticin 1
PTGS2	prostaglandin-Endoperoxide Synthase 2
SCNN1A	sodium channel, non-voltage gated 1 alpha subunit
SCNN1B	sodium channel, non-voltage gated 1 beta subunit
SCNN1G	sodium channel, non-voltage gated 1 gamma subunit
SCGB1A1	secretoglobin Family 1A Member 1

<i>SLC25A1</i>	solute carrier family 25 member 1
<i>SLC25A11</i>	solute carrier family 25 member 11
<i>SLC25A14</i>	solute carrier family 25 member 14
<i>SLC25A22</i>	solute carrier family 25 member 22
<i>TERT</i>	telomerase reverse transcriptase
<i>TIMP3</i>	tissue inhibitor of metalloproteinases 3
<i>TXN2</i>	thioredoxin 2
<i>ZNF319</i>	zinc finger protein 319

Chapter 1

Introduction

1.1 The Human Endometrium

The endometrium is the inner mucous membrane that lines the uterine cavity. This complex tissue provides a receptive environment for embryo implantation in the maternal organism. Many cell types take part in its composition. Simple columnar epithelial cells comprise the organ lumen coating, and the tubular glands extend from the surface epithelium to the endometrial-myometrial interface. Underlying the luminal epithelium, and surrounding the glands, the endometrial stroma consists of a balanced assortment of different cell types. Specialized fibroblasts that undergo mesenchymal-to-epithelial differentiation in every menstrual cycle are the main components (Brosens et al., 1999). Immune cells, such as natural killer cells, macrophages, T cells and granulocytes also reside in this endometrial compartment (Lee *et al.* 2011). Basal and spiral arteries with their endothelial cells and stem-like perivascular cells are also present in the stroma (Masuda et al., 2012). From the histophysiological aspect, the endometrium consists of two distinct layers. The superficial transient and dynamic functionalis, which is shed during menstruation, and the deeper persistent basalis, which contains the glandular fundi, and from which the post-menstrual regeneration process arises (Figure 1.1).

The plasticity of the endometrium is extraordinary, alternating regeneration, proliferation, differentiation and sloughing. The whole sequence occurs in mean 28 day intervals, and a woman is expected to have around 400 menstrual cycles during her reproductive life (Collins, 1985). These cyclic changes are tightly regulated by the secreted ovarian steroid hormones progesterone (P4) and oestradiol (E2). There is a gradient of responsiveness to these hormones across the endometrial layers, the upper portions undergoing marked progression of histological changes, while the basal compartment exhibits only subtle alterations.

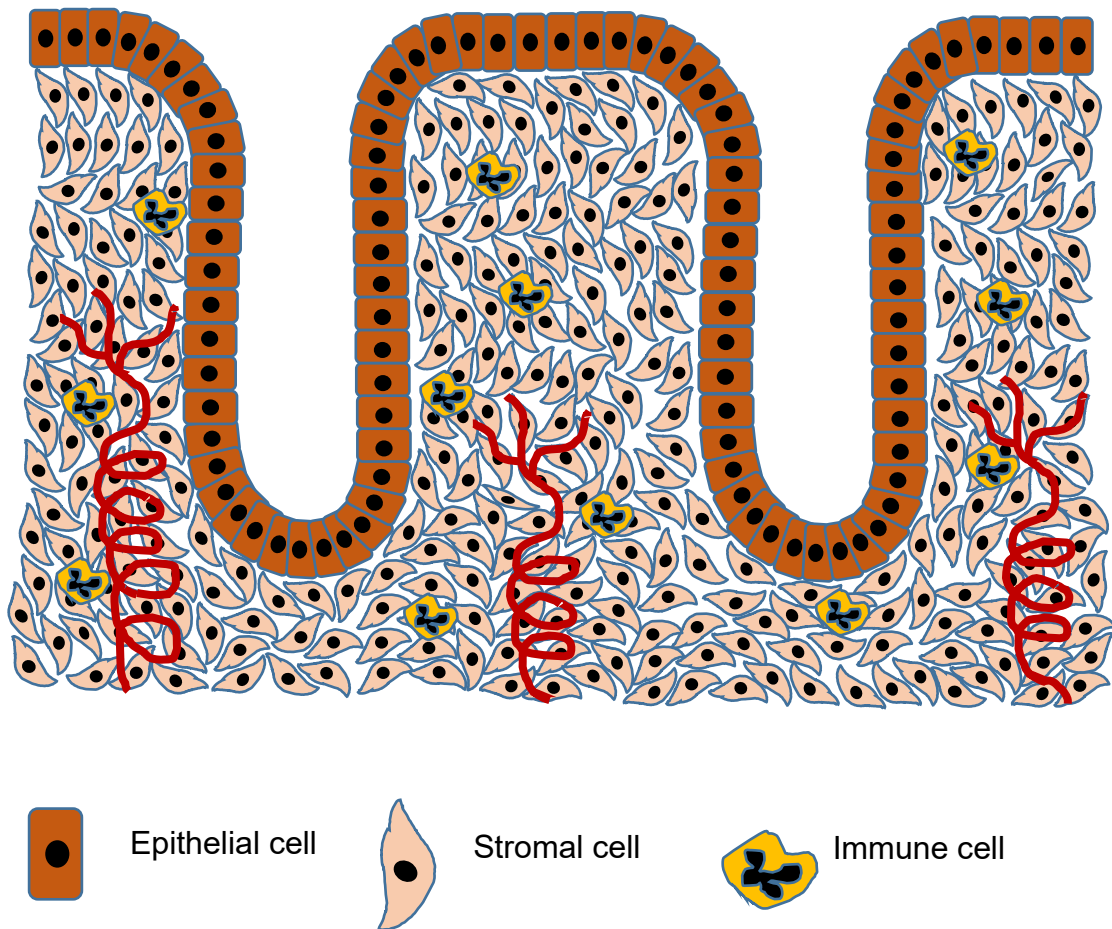


Figure 1.1 Cartoon of the endometrium with the different cell types and spiral arteries. The endometrium comprises the epithelial and stromal compartments.

The regeneration process is initiated by progenitor cells, through asymmetric division, producing the differentiated cells for uterine cavity reepithelization. The first evidence for the existence of human endometrial epithelial and stromal progenitor cells was described by Chan et al. (2004). The authors demonstrated the presence of 0.22 % clonogenic cells amongst the endometrial epithelial population and 1.25 % amongst stromal cells. Other properties of stem cells, such as self-renewal and pluripotency were also demonstrated later (Gargett et al., 2016). More recently, the identification of progenitor cell markers allowed the use of magnetic and fluorescent separation for their isolation and phenotyping (Masuda et al., 2012, Valentijn et al., 2013, Murakami

et al., 2014). Initially, it was believed the endometrial stem-like cells would reside exclusively in the basalis, but progenitor cell niches were also later detected in the functionalis (Schüring et al., 2011, Valentijn et al., 2013).

1.2 The Dynamics of Endometrium, During the Menstrual Cycle

The endometrium is one of the main target tissues of sex steroid hormones. Cycles of proliferation, differentiation, shedding and regeneration take place monthly, during the reproductive lifespan, in response to fluctuating serum levels of P4 and E2, with the purpose of preparing for embryo implantation. Specific changes occur in the epithelial and stromal compartments, and a striking cross-talk between these endometrial compartments is critical for the normal cycling and function. The length of the menstrual cycle is variable, and actually, only 10% of women have a typical 28 day cycle. The follicular phase accounts for most of the variability, ranging from 10 to 23 days (Mihm et al., 2011).

1.2.1 Proliferative Phase

The first day of menstrual bleeding is considered day 1 of the menstrual cycle. During the proliferative phase of the endometrium, which corresponds to the follicular phase of the ovaries, increasing levels of granulosa cell-synthesized E2 induce proliferation in glands and stroma. The thickness of the endometrium changes from less than 2 mm to 7 mm, from the beginning to the end of the proliferative phase (Hawkins and Matzuk, 2008). The regeneration is triggered by E2, and starts from the basalis. The luminal epithelium (LE) is completely restored by day 5, and no scarring is observed. The stromal and epithelial progenitor cells may contribute for this phenomenon (Gargett and Masuda, 2010).

In the early proliferative phase, glands are narrow, straight and tubular, and are comprised of low columnar epithelial cells. Later, they become tortuous and more

convoluted, columnar cells are higher, and sub-nuclear vacuoles are occasionally observed. Also in the late proliferative phase, the stroma is densely cellular, and by the pre-ovulatory period, oedema starts to develop in this compartment. Ciliation in focal areas on the luminal epithelium is also present, right before the secretory phase. E2-driven proliferation peaks between days 8 and 10. With the luteinisation of the dominant follicle, by increasing secretion of luteinizing hormone (LH), serum P4 levels rise, inhibiting the mitogenic effects of E2 in the pre-ovulatory period (Archer et al., 1991).

The actions of the sex steroids in the endometrium are carried out through binding of these ligands to their cognate nuclear receptors, oestrogen receptor (ER) and progesterone receptor (PR).

There are two isoforms of ER: ER α and ER β , encoded by two distinct genes. ER α is the predominant isoform expressed in the endometrium, and is sufficient to trigger endometrial proliferation (Hewitt et al., 2003). ER α is highly expressed in glands and stroma during the proliferative phase, with a dramatic decline in the secretory phase (Brenner et al., 1990). Upon ligand binding, ER α induces cell cycle progression by activation of the PI3K/AKT pathway and hyperphosphorylation of retinoblastoma protein (Rb) and p107 proteins, activating the DNA replication machinery for entry in the S-phase (Chen et al., 2005; Wang et al., 2007). The effects of E2 in epithelial proliferation is mediated by a paracrine action of stromal ER α (Cooke et al., 2007), while epithelial ER α is suggested to prevent apoptosis (Winuthayanon et al., 2010).

Increasing levels of E2 in the late follicular phase trigger the mid-cycle LH surge, leading to ovulation, which marks the beginning of luteal phase in the ovary and the secretory phase in the endometrium.

1.2.2 Secretory Phase

After ovulation, the dominant follicle, now known as the corpus luteum (CL), progressively increases P4 secretion in response to LH. The effects of P4 will prevail during the secretory phase, counteracting the mitogenic effects of E2. P4 also drives the striking changes observed in the stroma and the secretory function of the glands.

There are two isoforms of PR: PRA and PRB, both encoded by the same gene. PRA is the predominant functional variant in the endometrium. The expression of PR is higher during proliferative phase, markedly decreasing in secretory phase. *PR* expression is induced by E2 and inhibited by P4 (Moutsatsou and Sekeris, 2006). Ablation of murine PR resulted in endometrial epithelial hyperplastic response to E2 and P4 (Lyndon *et al.*, 1995; Kurita *et al.* 1998). Specific knockout of PRA also produced epithelial proliferation upon P4 treatment, and the proliferation was demonstrated to be dependent on PRB (Mulac-Jericevic *et al.*, 2000).

The anti-proliferative actions of P4 in the epithelial compartment occur in a paracrine manner, through stromal PR. Heart and neural crest derivatives expressed 2 (*HAND2*) is a P4 regulated gene in the stroma, and it downregulates fibroblast growth factor (*FGF*) expression. FGF binds to FGF receptor in the epithelial cells to activate extracellular signal-regulator kinase (ERK) 1 and 2 or the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathways, both involved in epithelial proliferation (Li *et al.*, 2011). Another indirect mechanism for P4 is the induction of 17 β -hydroxysteroid dehydrogenase type 2 (17 β -HSD2) in the epithelium, through binding to stromal PR. This enzyme catalyses the conversion of E2, a potent oestrogen, into oestrone, a weak form, thereby reducing the proliferative effects of E2 (Yang *et al.*, 2001). A direct effect on epithelial PR is also described in murine endometrium. Upon ligand binding, epithelial PR induces the expression of Krüppel-like factor (KLF) 15, which, in turn, negatively regulates E2-induced epithelial cell proliferation, by

inhibition of mini-chromosome maintenance protein 2 (Mcm2) expression (Ray and Pollard, 2012). MCM2 is required for initiation of DNA synthesis. Epithelial PR also affects stromal functions: at least in mice, the selective knockdown of epithelial *Pgr* resulted in infertile mice and lack of decidualization (Franco et al., 2012).

Accumulation of glycogen rich sub-nuclear vacuoles in the columnar cells of the glands can be detected in early secretory phase. Later, these vacuoles shift to a supra-nuclear position, and by the peak of secretory activity in the mid-secretory phase, they are lost into the glandular lumen and are absorbed by the embryo (McCluggage, 2011). A spherical stack of interdigitating nuclear tubules can be observed in 5% of the epithelial cells in the mid-secretory phase. This structure, named nucleolar channel system (NCS), has only been described in the endometrium. Its role is still elusive. It has been suggested that the blastocyst would select a cluster of luminal cells containing NCS for apposition (Isaac et al., 2001). An enlargement of mitochondria with prominent cristae (Armstrong et al., 1973) is also present in epithelial cells.

In the stromal compartment, oedema is marked, and pronounced changes develop in the mesenchymal cells, in the mid-secretory phase. The cells around the blood vessels are enlarged, present an eosinophilic cytoplasm, and an extracellular matrix (ECM) is easily identified. These alterations progressively extend to the sub-epithelial cells, and later, becomes a global event in the stroma. The changes are referred to as decidualization, and occur in response to the rising levels of serum P4 and local secretion of cyclic adenosine monophosphate (cAMP; Gellersen and Brosens, 2003). The stromal cells shift to a secretory phenotype. An influx of specialized uterine natural killer (uNK) cells, also starting around the blood vessels, is observed in the mid-secretory phase. This phase is the endometrial receptive period, when a supportive environment is created to accommodate the implanting blastocyst.

In the late secretory phase, serum levels of P4 gradually decrease, leading to regression of gland secretion and inflammation process in the stroma, culminating in menstruation.

1.2.3 Menstruation

If pregnancy does not ensue, CL function declines, resulting in decreasing levels of P4 in the late secretory phase. That triggers the cascade of molecular and cellular interactions that result in epithelial and stromal breakdown, and eventually, menstruation. There is glandular collapse, bestowing them a serrated appearance. The stroma is condensed, presenting tight aggregates of stromal cells with hyperchromatic nuclei, the so called 'stromal blue balls' (McClugagge, 2011). Decidual cells lose their enlarged round features. An influx of neutrophils and monocytes can be observed in the premenstrual period, along with interstitial haemorrhage and fibrin deposition, and uNK cells undergo apoptosis. In the glands, both the NCS and the giant mitochondria disappear (McClugagge, 2011). Inflammation is initiated by activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pathway, with increased secretion of several mediators: interleukin (IL) 8, monocyte chemotactic protein 1 (MCP1), cyclooxygenase 2 (COX₂), IL6, IL1, IL33, granulocyte-macrophage colony-stimulating factor (GM-CSF) (Evans and Salamonsen, 2014). Matrix metalloproteases (MMP) are mainly secreted by the infiltrating neutrophils. The enzymes catabolize ECM, collaborating in stromal collapse. Vasoconstriction of spiral arteries is induced by prostaglandin F₂ α (PGF₂ α) and endothelins. Fibrinolysis is activated in the late secretory phase. Tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) are up-regulated and their inhibitor PAI1 (plasminogen activator inhibitor 1) is downregulated during the period. Apoptosis is observed in the functionalis, with high expression of caspases 3, 8 and 9, and translocation of forkhead box protein O1 (FOXO1) to the nucleus. Conversely, BCL-2, an anti-apoptotic molecule, is expressed in the basalis

(Henriet et al., 2012). Interestingly, exogenous P4 can only reverse the process within the first 36 hours of P4 withdrawal. This suggests that menstruation is initiated by P4 withdrawal-induced vasoconstriction and inflammatory response. However, in a second phase, the released chemokines attract the leukocytes which secrete MMP, leading to ECM breakdown and tissue collapse (Slayden and Brenner, 2006). Another remarkable fact is that spontaneous decidualization always precedes the cyclic shedding of the endometrium in menstruating species, linking these two phenomena (Brosens and Blanks, 2013).

Normal function of the coagulation system is required to cease the menstrual bleeding. Tissue factor-derived thrombin and von Willebrand factor interaction with platelet glycoproteins act together to form the fibrin plugs that prevent excessive blood loss (Davies and Kadir, 2012).

The inflammation must be self-limiting to avoid excessive tissue injury. The pro-inflammatory cytokine IL1 induces expression of 11 β hydroxysteroid dehydrogenase-1 (11 β HSD1). This enzyme catalyses the conversion of cortisone into cortisol, a glucocorticoid that is a potent inhibitor of the inflammatory response. The glucocorticoid receptor is expressed in the stromal compartment, including leukocytes and endothelial cells. MMP activity is also limited during the menstruation to avoid uncontrolled local damage. Tissue inhibitors of MMP (TIMPs), protease inhibitor α_2 -macroglobulin and low-density lipoprotein receptor-related protein-1 (LRP1) are involved in the repression of MMP activity (Maybin and Critchley, 2015).

After the tissue detachment and sloughing of the functionalis, regeneration must be attained. Reepithelization of the endometrial surface and blood vessel regrowth are the main events involved in the endometrial repair. Stem-like cells in the gland stumps of the basalis are likely to be involved in the restoration of the surface epithelium (Gargett et al., 2016), but mesenchymal-to-epithelial transition has also been described (Gary et al., 2009, Patterson et al., 2013). This initial process is

independent of oestrogen stimulation (Bigsby, 2002, Kaitu'u-Lino et al., 2010). Blood vessel repair involves hypoxia-inducible angiogenic factors, such as hypoxia-inducible factor 1 (HIF1), angiopoietins, vascular epithelial growth factor (VEGF), platelet-derived growth factor (PDGF) and FGF, some of them known to have mitogenic effects on endometrial epithelial cells (Jabour et al., 2006, Maybin et al., 2011).

With the regeneration of the functionalis layer and rising levels of E2 secreted by the ovarian follicles, endometrial proliferation is resumed, and a new menstrual cycle begins.

1.3 Human embryo implantation

Implantation is a complex process comprising a highly co-ordinated crosstalk between the embryo and the uterus, as well as communication amongst the endometrial compartments (epithelium, stroma and, possibly, myometrium). This phenomenon is believed to involve a precisely timed arrival of a competent blastocyst into a uterine cavity lined by a receptive endometrium. Due to ethical concerns and technical limitations, most of our knowledge on this issue derives from animal research, predominantly from mouse knock-out studies.

1.3.1 Embryo Implantation Steps

After oocyte fertilization, exponential cell division occurs simultaneously with establishment of cell polarity and compaction of blastomeres, to produce a morula. Between 4 and 5 days after fertilization, the development of a cavity within the cell bulk, and the differentiation of two cell lineages, the outer specialized trophoectoderm and inner cell mass (ICM), defines the blastocyst phase. The late phase blastocyst develops two other cell types from the ICM: the epiblast and the primitive endoderm (Cockburn and Rossant, 2010). Next, the blastocyst hatches from the zona pellucida,

before initiating the implantation process. Serine proteases seem to be crucial for hatching (Lin et al., 2001, O'Sullivan et al., 2001). The blastocyst enters the endometrial cavity, approximately 5 days after fertilization, and the phases of implantation unfold (Wang and Dey, 2006).

1.3.1.1 Apposition

Before initial adhesion to the luminal epithelium, the human blastocyst orients itself with the trophoectoderm overlying the ICM apposing to the endometrium. The embryo approaches and becomes loosely adhered to the uterine wall on the implantation site. At this stage in mice, intact blastocysts can be retrieved by uterine flushing (Su and Fazleabas, 2015). The dialogue between conceptus and endometrium drives this sequence. Many chemokines such as monocyte chemoattractant protein 1 (MCP1), IL8 and regulated on activation, normal T cell expressed and secreted (RANTES) are produced by the blastocyst during this phase (Caballero-Campo et al., 2002). L-selectin mediates initial adhesion to the endometrial epithelium through MECA-79 and HECA 452, its carbohydrate ligands (Fukuda and Sugihara, 2008). MUC1 acts as a scaffold for L-selectin ligands. MUC1 is an anti-adherent glycoprotein present on the epithelial surface of the endometrium, inhibiting cell-cell and cell-matrix adhesion. MUC1 is up-regulated in humans during the mid-secretory phase. However, it was demonstrated that it disappears at the implantation site (Meseguer et al., 2001, Thathian and Carson, 2004). Some proteases (MMP14 and ADAM17) are involved in this clearance of apical surface MUC1 (Aplin, 2006). Trophoblasts express heparin-binding EGF-like growth factor (HB-EGF) and its receptor, ErbB4. In a paracrine and juxtacrine mode, embryo-derived HB-EGF induces its own expression in the luminal epithelium. Epithelial-derived HB-EGF, in turn, triggers trophoblast modifications required for apposition and the following steps. Another molecule crucially involved in the initial adhesion of the embryo is trophinin. This transmembrane protein expressed by trophoblasts mediates homophilic cell adhesion via trophinin-trophinin binding.

Human chorionic gonadotrophin (hCG) stimulates trophinin synthesis by luminal epithelial cells of implantation site, which bind to trophoblastic cells expressing the same protein (Fukuda and Sugihara, 2008).

1.3.1.2 Attachment

During this integrin-dependent phase, the blastocyst attaches more stably to the maternal surface. Many adhesion molecules expressed in the trophoblasts and the luminal epithelium are involved in the coupling process. Integrins are plasma membrane glycoproteins comprising heterodimeric α and β subunits. They bind to other cells and to ECM ligands, such as osteopontin, fibronectin, vitronectin, heparan sulphate proteoglycans and laminin (Bazer et al., 2009). Osteopontin contain the integrin binding RGD motif, and is up-regulated by P4 in the epithelial compartment and trophoblasts. The blastocyst induces integrin expression in the endometrial surface. These receptors also work as signal transduction factors, driving cytoskeleton remodelling. Some adhesion molecules, such as basigin and activated leukocyte cell adhesion molecule (ALCAM), activate MMPs, triggering the ECM remodelling necessary for blastocyst invasion (Singh and Aplin, 2009).

1.3.1.3 Invasion

After attachment, the blastocyst must cross the endometrial surface epithelial barrier, and invade the underlying stroma. Different mechanisms have been proposed for this breaching process. Penetration of the trophoblast through gaps between neighbouring luminal epithelial cells, defined as interstitial implantation, was inferred based on histological findings in the implantation site (Bischof and Campana, 1996). Epithelial cell apoptosis induced by trophoblast FAS ligand interaction with epithelial FAS receptor or mediated by trophinin has also been suggested (Boeddeker and Hess, 2015). Uchida et al. (2012) demonstrated epithelial-to-mesenchymal transition of Ishikawa cells, a human endometrial epithelial cancer cell line, induced by JAR

cells, a human choriocarcinoma cell line, postulating that this transformation would underpin the embryo invasion. After breaching the surface epithelium, a thin layer of collagen type IV, the basal lamina, is ruptured. The stromal matrix is the last obstacle to be transposed. During penetration, trophoblast cells proliferate, differentiate and fuse to form the syncytiotrophoblasts. These cells secrete MMPs that degrade the stromal ECM (Wang et al., 2001). By day 10 after fertilization, the blastocyst is completely embedded in the stroma and overlaid by a newly grown luminal epithelium. The decidualized stromal cells, through a constant cross-talk with the blastocyst, control trophoblast invasion and indeed participate actively in the process of surrounding the conceptus (Gellersen et al., 2010). The endometrial arteries are also invaded by trophoblasts to establish the haemochorial placenta. However, the maternal-foetal circulation is only thoroughly functional, having blood from spiral arteries flowing to the lacunar spaces, by the end of the first trimester of pregnancy. Before that, aggregates of trophoblasts plug the distal segments of the arteries. Table 1.1 summarizes some of the embryonic signals and their roles in implantation.

Table 1.1 Embryo signals and roles in implantation

Embryo signal	Role	Reference
hCG	<ul style="list-style-type: none"> - Corpus luteum rescue - Trophoblast differentiation - Trophoblast migration - Endometrial gland differentiation - Induction of LIF, IL6, IL11, MMP7, VEGF, CXCL14, GM-CSF, PGE₂ in endometrium - Decidualization 	<p>Järvelä et al., 20098</p> <p>Shi et al., 1993</p> <p>Zygmunt et al., 2005</p> <p>Fazleabas et al., 1999</p> <p>Banaszak et al., 2000</p> <p>Paiva et al., 2011</p> <p>Kasahara et al., 2001</p>
EGF	<ul style="list-style-type: none"> - Syncytialization 	Dakour et al., 1999
Platelet activating factor (PAF)	<ul style="list-style-type: none"> - Embryo development 	Stoddart et al., 1996
Pregnancy-associated plasma protein A (PAPPA)	<ul style="list-style-type: none"> - Embryo development and adhesion 	Wang et al., 2014
IL1α	<ul style="list-style-type: none"> - Adhesion (induction of integrin β3) 	Simon et al., 1997
IL1β	<ul style="list-style-type: none"> - Decidualization 	Strakova et al., 2005
Preimplantation factor (PIF)	<ul style="list-style-type: none"> - Adhesion, decidualization 	Barnea et al., 2012
HB-EGF	<ul style="list-style-type: none"> - Apposition - Embryo development 	<p>Jessmon et al., 2009</p> <p>Wang et al., 2000</p>

Trophinin	- Adhesion	Fukuda and Sugihara 2008
L-selectin	- Adhesion	Genbacev et al. 2003
Integrins	- Adhesion	Burrow et al. 1993
Proteases	- Decidualization	Ruan et al., 2012
	- Embryo selection	Brosens et al., 2014
hPL	- Endometrial gland secretion	Noel et al., 2003
WNT	- Trophoblast invasiveness	Pollheimer et al., 2006

1.3.2 Endometrial Preparation

In order to allow the blastocyst to implant, the endometrium must shift to a receptive phenotype. The endometrial receptive phase is also known as the window of implantation (WOI), and occurs in the secretory phase of the menstrual cycle, spanning day 20 to day 24 of a standard 28 day menstrual cycle, or 6 to 10 days after the LH surge (van Mourik et al., 2009). Morphological changes in the endometrium are observed during this period, as described in section 1.2.2. Protrusions on the apical membrane of luminal epithelial cells, defined as pinopodes or uterodomes, have been described. These variable-sized bleb-like structures appear for around 48 hours, in the mid-secretory phase, and were reported as a preferential site for blastocyst-epithelial physical interaction (Quinn and Casper, 2009).

The acquisition of receptivity depends chiefly on coordinated and sequential exposure to oestrogen and P4 from the beginning of the menstrual cycle. These sex steroid hormones act mainly binding to their respective nuclear receptors, and recruiting co-chaperones and co-regulators. The resulting transcription machinery mobilizes

several molecular modulators in a spatiotemporal mode, affecting various signal transduction pathways. The steroid hormones also exert their effects in non-canonical manners, binding to G protein-coupled receptors in the plasma membrane, through interaction of the nuclear receptors with other transcription factors, or affecting mRNA stability (Young, 2013). Oestrogen is crucial for endometrial proliferation, but its role in receptivity in humans is controversial. Negative effect on *in vitro* fertilization implantation rates has been indeed demonstrated (Shapiro et al., 2011). P4, in turn, is absolutely essential and sufficient for implantation and early pregnancy survival, as proved by inhibition of its biosynthesis and use of anti-progestins (Rashid et al., 2012). Signals from the embryo are also involved in endometrial receptivity attainment. hCG flushed into the uterine cavity triggers the endometrial expression of various genes involved in implantation (Sherwin et al., 2007, Paiva et al., 2011). Human embryo conditioned media have similar effects in human endometrial epithelial cells (HEEC) and in mouse uterus (Cuman et al., 2013, Brosens et al., 2014). A vast repertoire of molecules and their receptors steer the modifications taking place in the endometrium during the window of implantation. Cytokines (LIF, IL6, IL1, IL11, IL18, prostaglandins, TNF α , CSF1, osteopontin), chemokines (CX3CL1, CCL7, CCL14, CCL4), leptin, IHH, WNT members, growth factors (HB-EGF, IGFs, TGF β , EGF), morphogens (HOXA10 and 11, MSX1 and 2) and the aforementioned adhesion molecules act in synchrony with signals relayed from the embryo to create a supportive environment for the arriving blastocyst (Dimitriadis, 2005, van Mourik et al., 2009). The role of some of these factors are summarized in Table 1.2.

Table 1.2 Factors involved in endometrial receptivity and the roles in implantation.

Factor	Roles	References
COX2	- Knockout mice exhibit implantation failure.	Lim et al., 1997
cPLA2α	- Releases arachidonic acid for prostaglandin synthesis. - Null mice exhibit implantation defects that are rescued by exogenous PGE ₂ and PGI ₂ .	Song et al., 2002
FKBP52	- P4 co-chaperone. - Knockout mice exhibit implantation failure and excessive epithelial proliferation.	Yang et al., 2006
HB-EGF	- Apposition. - Trophoblast differentiation (invasion). - Knockout mice show implantation failure.	Xie et al., 2007 Wang et al., 2000
HOXA10/11	- Knockout mice show implantation failure, lack of decidualization and absence of Lif expression.	Gendron et al., 1997 Das, 2010
IHH	- Implantation and decidualization. - Control of epithelial proliferation by paracrine signalling to stroma.	Kurihara et al., 2007
IL1	- Regulates $\beta 3$ integrin expression. - Induces Lif and leptin expression in endometrium and hCG in trophoblast.	Dimitriadis et al., 2005 Gonzalez et al., 2004
IL6st (Gp130)	- Dimerises with IL6 receptors to phosphorylate STAT3. - Secretion of soluble form is reduced in infertile women during WOI.	Ernst et al., 2001 Sherwin et al., 2002
IL11	- Reduced peri-implantation epithelial expression in recurrent miscarriages. - Required for decidualization.	Linjawi et al., 2004 Robb et al., 1998

Leptin	- Interacts with IL1R to induce Lif and $\beta 3$ integrin expression.	Gonzalez et al., 2004
LIF (IL6 family)	<ul style="list-style-type: none"> - Adhesion and decidualization. - Growth and differentiation of trophoblast (invasion). - Regulated by P4, hCG, TGFβ, IL1, HB-EGF, PDGF TNFα. - Inhibit Msx1 expression. - Knockout mice show implantation failure and lack of decidualization. - Patients with multiple implantation failures show lower levels, in secretory phase. 	<p>Salleh and Giribabu, 2014</p> <p>van Mourik et al., 2009</p> <p>Stewart et al., 1992</p> <p>Song et al., 2000</p> <p>Hambartsoumian, 1998</p>
MSX1/2	<ul style="list-style-type: none"> - Paracrine signalling between epithelium and stroma. - Knockout mice exhibit implantation failure and impaired luminal polarity. 	<p>Daikoku et al., 2011</p> <p>Nallasamy et al., 2012</p>
MUC1	<ul style="list-style-type: none"> - Anti-adhesion molecule. - Up-regulated during WOI, but locally cleared at implantation site by blastocyst. 	<p>Messeger et al., 2001</p> <p>Thathiah and Carson, 2004</p>
P53	- Knockout mice exhibit implantation failure which is rescued by exogenous Lif.	Hu et al., 2007
SRC2	<ul style="list-style-type: none"> - Co-activator of PR. - Knockout mice exhibit implantation failure. 	Mukherjee et al., 2002
TGFβ	- Induces VEGF expression in trophoblast.	Chung et al., 2000
WNT4	- Knockout mice exhibit implantation and decidualization defects.	Franco et al., 2011
WNT7a	- Knockout mice are devoid of uterine glands, show reduced Lif, and fail to implant.	Dunlap et al., 2011

There is scarce information about the factors defining the post-receptive phase, or the closure of the WOI. Fall in P4 levels triggers a cascade of events that culminate in menstruation, whereas hCG rescues CL from luteolysis, preventing decrease in P4 secretion and excessive inflammation (Tabibzadeh, 1998, Salker et al., 2012).

Impaired receptivity is considered an important cause of infertility and a limiting factor in assisted reproductive technology success (Fatemi and Popovic-Todorovic, 2013). Consequently, multiple approaches for assessing endometrial receptivity have been developed. One of the first attempts to define the histological changes in the endometrium occurring along the menstrual cycle was described by Noyes et al, in 1950. However this method proved to be inadequate due to low reproducibility and to not correlate with infertility (Díaz-Gimeno et al., 2013). Visualization of pinopodes was also described as a method to determine the WOI (Nikas et al., 1995). Besides the unavailability of electron microscopes for wider use, these structures are not exclusive to the mid-secretory phase, being identified during the whole secretory phase and even in pregnancy (Quinn and Casper, 2009). Analysis of individual markers such as LIF, CSF1, IL1, MUC1, trophinin, L-selectin ligands and integrins were not validated to be used for this purpose (Lessey, 2011, Malhotra et al., 2012). The echographic appearance, thickness and blood flow of endometrium evaluated through transvaginal ultrasound is currently used, but also demonstrates a disappointing performance for clinical use (Schild et al., 2001).

1.4 Stromal Decidualization

In addition to all the remodelling taking place in the luminal epithelium during the mid-secretory phase, remarkable changes occur in the underlying stroma. This transformation is essential to accommodate the blastocyst and ensure the development of a healthy pregnancy.

Decidualization is the mesenchymal to epithelial transition of the endometrial fibroblasts, arising in the mid-luteal phase, and conferring on them a specialized secretory phenotype. This phenomenon is observed in all the species in which the embryo breaches the luminal epithelium and invades the stromal compartment. However, in contrast with most species, decidualization in humans unfolds in the mid-luteal phase, independently of the blastocyst presence. The earliest sign of decidualization is stromal oedema, starting around day 18 of the cycle. Morphological changes can be detected in the cells surrounding the spiral arteries around day 23. Next, these modifications span the cells underlying the luminal epithelium, later becoming a global stromal feature (Gellersen et al., 2007). The spindle-shape morphology is replaced by a round configuration due to cytoplasm increase (Figure 1.2). At sub-cellular level, expansion of the endoplasmic reticulum and Golgi apparatus, and accumulation of glycogen and lipid droplets in the cytoplasm are present. Projections of cell surface extending into the ECM or indenting into neighbouring cells can be detected. An influx of immune cells can be later observed in response to local production of chemokines. Profound changes in ECM volume and composition are characteristic of this process. The expanding matrix show an increase in collagen type IV and laminin (Gellersen and Brosens, 2014).

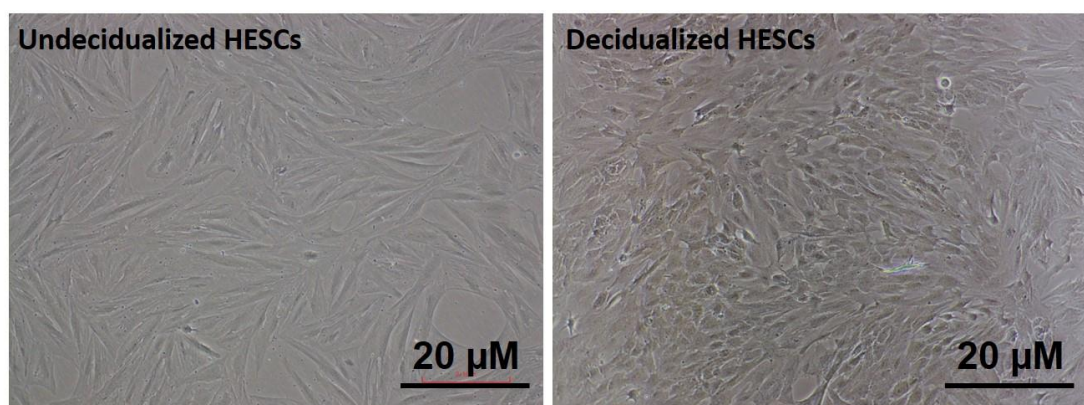


Figure 1.2 Decidualizing human endometrial stromal cells (HESCs). A confluent HESC culture before decidualization (left panel), and after two days of treatment with 0.5 mM cAMP and 1 μ M medroxyprogesterone acetate (MPA) (right panel).

1.4.1 Decidualization Markers

Prolactin (PRL) and insulin-like growth factor binding protein 1 (IGFBP1) are massively enriched in amniotic fluid and their source was discovered to be the decidua (Riddick et al., 1978, Rutanen et al., 1986). These factors are established as decidual phenotype markers, and are widely used to assess decidualization in endometrial stromal cell cultures (Gellersen and Brosens, 2014).

1.4.2 Mechanisms of Decidualization

1.4.2.1 P4 Signalling

The postovulatory rise of P4, secreted by the CL, is the main cue for stromal differentiation. As aforementioned, P4 acts through binding and activation of its cognate nuclear receptors. PRA seems to be a truncated version of the more transcriptionally active PRB (Vegeto et al., 1993). The two isoforms actually present distinct transactivational effects, specific to the cell type. While PRB is pivotal for mammary gland morphogenesis, PRA is critical for endometrial function (Mulac-Jerivic et al., 2000, Mulac-Jerivic et al., 2003). PRA knockout mice were unable to develop stromal decidualization. By contrast, selective ablation of PRB confirmed that PRA is necessary and sufficient to mediate endometrial P4 responses (Mulac-Jerivic et al., 2000).

PR is part of a transcription complex assembly, being associated with several chaperone proteins, such as heat-shock proteins (HSP40, HSP70, HSP90 and immunophilins (FK506 Binding Protein [FKBP] 4 and 5). These chaperone proteins maintain a conformational structure that makes the ligand binding domain accessible (Gellersen and Brosens, 2003). Upon P4 binding, the receptor changes its conformation, leading to phosphorylation, dissociation from the chaperones, dimerization, binding to responsive elements in promoter regions of the target genes,

and recruitment of co-activators SRC2, cAMP response element binding protein binding protein [CBP], p300/CBP associated factor [pCAF] and coactivator associated arginine methyltransferase 1 [CARM1]) (Wardell et al., 2002, Szwarc et al., 2014). The transcriptional machinery is complemented by interaction with factors induced by cAMP (Gellersen and Brosens, 2014), and dissociation of co-repressors (nuclear receptor co-repressor 1 [NCOR], nuclear receptor co-repressor 2 [SMRT]) (Wagner et al., 1998), resulting in transcription initiation.

The first morphological changes associated with decidual transformation can be detected approximately 10 days after the postovulatory rise in P4 levels (Noyes et al. 1950), indicating that the expression of decidua-specific genes is unlikely to be under direct control of activated PR. The fact that HESCs in culture require, at least, 8 days of treatment with P4 or a progestin to secrete detectable levels of PRL, corroborates the idea of an initiation factor for decidualization, other than P4 (Gellersen and Brosens, 2003).

1.4.2.2 cAMP Signalling

Cyclic AMP is produced upon ligand binding to G protein-coupled receptors. The α subunit is released from heterotrimeric $\alpha\beta\gamma$ G-protein complex, following binding of ligand to the receptor. Next, α activates the enzyme adenylyl cyclase (AC), which, in turn, synthesizes cAMP from adenosine triphosphate (ATP). This small molecule acts as a second messenger, activating the cAMP-dependent protein kinase (PKA). PKA complex is comprised of two regulatory and two catalytic subunits. The catalytic subunits are dissociated from the complex, and activated upon binding of cAMP to the regulatory subunits. Activated PKA phosphorylates target proteins in the cytoplasm or diffuses into the nucleus to modulate the activity of transcription factors (Fimia and Sassone-Corsi, 2001). The nuclear factors cAMP response element binding protein (CREB) and cAMP response element modulator (CREM) are

targets for PKA activation through phosphorylation. Once activated, CREB and CREM dimerize and bind to c-AMP response element (CRE) in the promoter region of target genes, recruiting the co-activator CBP. An alternative *CREM* promoter encodes inducible cAMP early repressor (ICER), which functions as a potent repressor of cAMP-induced transcription (Sassone-Corsi, 1998). Cyclic nucleotide phosphodiesterases (PDEs) are enzymes that degrade cAMP. The intracellular levels of the cyclic nucleotide are regulated by the balance between the activities of ACs and PDEs.

Huang et al. (1987) showed that PRL induction by HESCs treated with E2 and medroxyprogesterone acetate (MPA) was further enhanced by the addition of relaxin. Based on that observation, and on the fact that relaxin up-regulates cAMP in the endometrium, Tang et al. (1993) treated HESCs with forskolin and cAMP and observed PRL induction at mRNA, protein and secretion level. Ever since, a great amount of effort has been applied to unravel the role of this second messenger in endometrial decidualization. Many GPCR ligands, such as relaxin, PGE₂, hCG, LH, follicle stimulating hormone (FSH) and corticotropin releasing hormone (CRH) are involved in the rise of intracellular cAMP levels in HESCs, through activation of adenylyl cyclase (Fei et al., 1990, Yee and Kennedy, 1991, Tang and Gurpide, 1993, Makrigiannakis et al., 1999). cAMP signalling is controlled by a balance between synthesis and mechanisms that operate to terminate its effects (Sassone-Corsi, 2012). Decidualizing HESCs act in such a manner to ensure continuous stimulation of this pathway, in order to maintain the decidual phenotype. Some suggested mechanisms to sustain cAMP effects in HESCs, include: inactivation of PDE by tyrosine kinase (Bartsch and Ivell, 2004); alterations in the composition of PKA holoenzyme, shifting the ratio catalytic:regulatory subunits towards the catalytic subunits (Telgmann et al., 1997); and the recruitment of the co-activator CBP (Smith et al., 1996).

The actions of cAMP in decidualizing HESCs are mediated by some important downstream effectors. The CCAAT/enhancer-binding protein β (C/EBP β) is a key mediator of cAMP-induced transcription of *PRL* (Pohnke et al., 1999). FOXO1 interacts with C/EBP β to activate *PRL* promoter (Christian et al., 2002). cAMP induces phosphorylation, dimerization and nuclear translocation of signal transducer and activator of transcription 5 (STAT5) in a Janus kinase (JAK)-independent way. This transcription factor enhances activity of the *PRL* promoter in the same region as C/EBP β and FOXO1 (Mak et al., 2002).

1.4.2.3 Convergence of P4 and cAMP Signalling

Accumulated evidence points to an intricate cross-talk between PR and cAMP signal transduction pathways during the decidualization process. HESC cultures only maintain high sustained levels of *PRL* secretion, if treated with both progestin and cAMP analogue (Brosens et al., 1999). P4 potentiates PGE₂-promoted production of cAMP in HESCs (Houserman et al., 1989). Onapristone, an antiprogestin, inhibited cAMP-induced *PRL* expression in HESCs (Brosens et al., 1999). Several transcription factors induced by cAMP (C/EBP β , FOXO1, STAT5 and STAT3) interact with PR in the *PRL* promoter (Christian et al., 2002, Mak et al., 2002, Takano et al., 2007). cAMP has been demonstrated to disrupt the interaction between PR and the co-repressors NCOR and SMRT, enabling the recruitment of co-activators (Wagner et al., 1998). Sumoylation is a post-translational modification of proteins by the covalent attachment of a small ubiquitin-related modifier (SUMO). cAMP triggers global decrease in sumoylation and redistribution of SUMO-1 modified proteins in decidualizing HESCs. Loss of PRA sumoylation results in increased transcription activity (Jones et al., 2006).

In a concerted operation with cAMP pathway, PR function as a platform for assembly of a transcription complex. This transcription machinery, recruiting co-activators, and

interacting with other transcription factors, controls the expression of a decidual specific genotype (Figure 1.3).

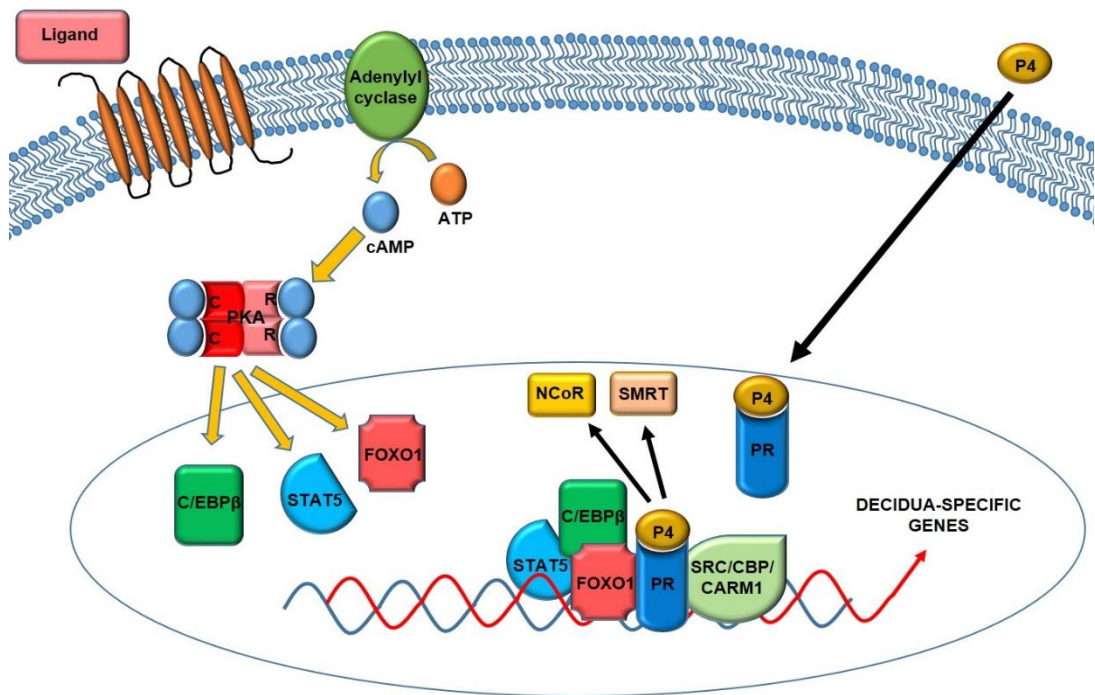


Figure1.3 Convergence of P4 and cAMP signalling in HESC decidualization. Ligand-bound progesterone receptor (PR) form a complex with co-activators (SRC, CBP, CARM1) and transcription factors induced by cAMP signalling (C/EBP β , STAT5 and FOXO1), and is dissociated from co-repressors (NCoR and SMRT). The transcription machinery activates the promoter of decidual-specific genes.

1.4.3 Roles of Decidualization

1.4.3.1 Haemostasis

Human implantation requires intrusion into the endometrial stroma by the blastocyst, along with extensive remodelling of the uterine vasculature by the trophoblast and uNK cells (Chakraborty et al., 2012). This invasive process may lead to rupture of the endometrial vasculature. The risk of haemorrhage is counteracted by haemostatic mechanisms provided by the decidualized stroma.

Tissue factor (TF) is a type 2 cytokine receptor, considered the primary initiator of the extrinsic pathway of coagulation. It acts as a receptor for blood coagulation factor VII. TF is constitutively expressed at perivascular sites, but not in endothelial cells. TF/VIIa activates factors X and IX, ultimately leading to activation of thrombin (Lockwood et al., 2009) (Figure 1.4). Thrombin cleaves protease activated receptors (PARs). These receptors have a tethered ligand, which is released for binding by protease cleavage. While trypsin activates PAR2, thrombin acts on PAR1, 2 and 3 to convert fibrinogen into fibrin and promote platelet aggregation (Coughlin, 2000). Thrombin also induces secretion of soluble fms-like tyrosine kinase 1 (sFlt1) by dendritic cells from first trimester decidua. This soluble receptor binds to the angiogenic factors VEGF and placental growth factor (PIGF), preventing them to interact with the active membrane bound isoform of Flt1 (Lockwood et al., 2007).

Being induced in HESCs by a concerted action of cAMP and P4, TF has been used as a decidualization marker (Lockwood et al., 1993, Christian et al., 2001). The stimulatory effect of P4 on TF was suggested to be modulated by interaction of PR and epithelial growth factor receptor (EGFR) signalling, leading to phosphorylation of the transcription factor SP1 (Lockwood et al., 2000). The same interaction (PR/EGFR signalling) is involved in up-regulation of PAI1 in HESCs. PAI1 inhibits tPA and uPA, impeding clot degradation (Lockwood, 2001).

These observations indicate that decidualization prevents bleeding at implantation by activating haemostatic mechanisms and by control of blood vessel remodelling and embryo invasion.

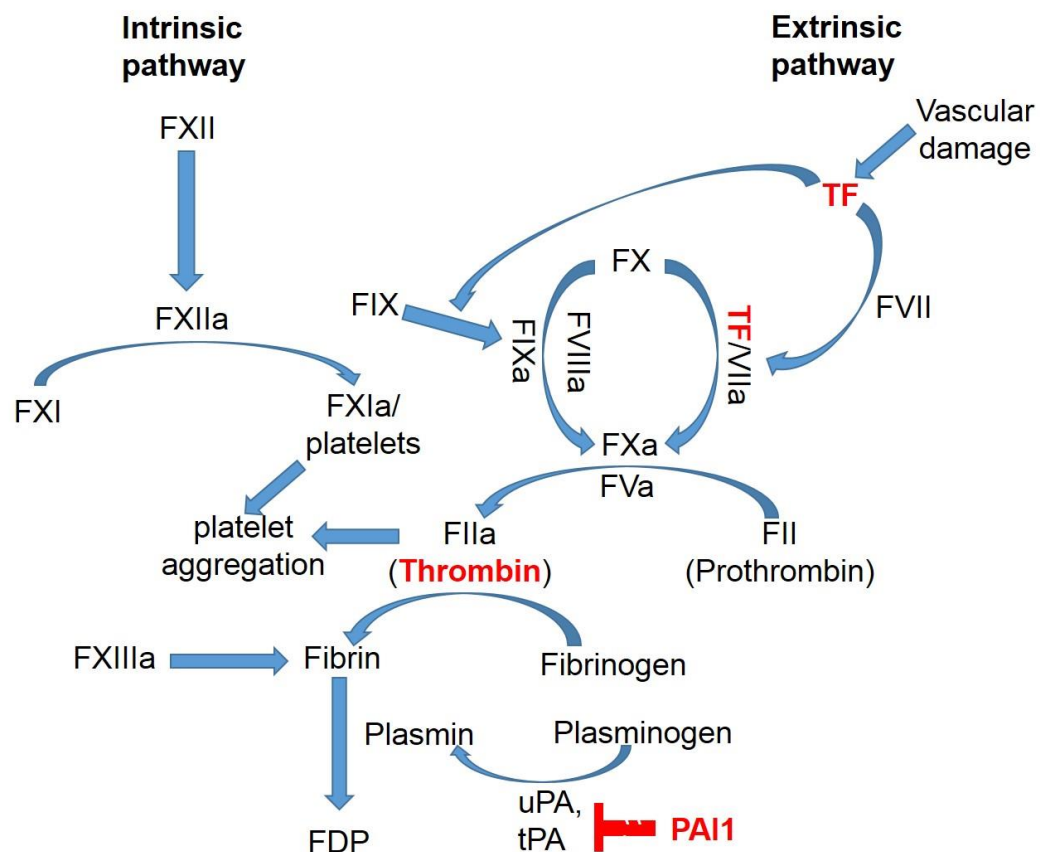


Figure 1.4 The coagulation cascade. Upon vascular damage, tissue factor (TF) comes into contact with its ligand, factor VII (FVII). The complex TF/FVIIa ultimately activates thrombin, which triggers platelet aggregation and fibrin production to generate a clot. The fibrin clot is degraded by plasmin, resulting in fibrin degradation products (FDP). Plasminogen activator inhibitor 1 (PAI1) inhibit the conversion of plasminogen to plasmin.

1.4.3.2 Control of Trophoblast Invasion

Human implantation is characterized by deep penetration of the embryo in the endometrium, and even in the stromal-myometrium border, termed junctional zone (Brosens et al., 2002). Trophoblast cells share some striking similarities with cancer cells, being highly proliferative, extremely invasive, immunologically tolerated by the host, and infiltrating the local vasculature (Ferretti et al. 2007). This remarkably invasive nature of the trophoblast must be tightly controlled in a temporal and spatial manner, to meet the embryo demand at the same time that the maternal integrity is safeguarded.

Embryo invasion involves proteolytic degradation and remodelling of the stromal ECM. In order to limit this process, a fine balance between the activity of MMPs and the opposing effect of TIMPs, both produced by the trophoblast and the decidualized stroma, must be achieved. TIMP3 was induced in HESC when co-cultured with first trimester trophoblast implants (Popovici et al., 2006). Decidualizing cells secrete TGF β , which, in turn, inhibits the synthesis of MMPs. During decidualization, HESCs also secrete the proteoglycan decorin into the ECM. Decorin, besides acting as a TGF β repository, inhibits proliferation, migration and invasiveness of extravillous trophoblast (EVT) cells (Xu et al., 2002). Plasmin is a serine protease, also involved in ECM remodelling. It is produced from its precursor, plasminogen, by action of tPA and uPA. PAI1 is up-regulated upon decidualization and inhibits the activity of plasminogen activators, reducing the synthesis of plasmin (Lockwood, 2001).

Decidualized stroma and ECM express adhesion molecules that contribute to limit trophoblast invasion (Burrows et al., 1996). CD38 is a tetraspanin expressed in the decidualized cells surrounding the embryo. This tumour suppressor interacts with other tetraspanins, integrins and E-cadherin, being engaged in cell adhesion, motility and invasion (Zhang et al., 2012). Elastin microfibril interface 1 (EMILIN1) is produced

by decidual HESCs in a gradient of increasing concentration towards the perivascular sites. This is consistent with the directional migration of EVT. Interaction between trophoblast and EMILIN1 is carried out via $\alpha 4/\beta 1$ integrin (Spessotto et al., 2006).

More recent evidence suggests that the maternal decidua, rather than acting as an inert tissue awaiting for invasion, plays an essential role in the encapsulation of the conceptus. The chemokine CXCL12, secreted by the trophoblast induces expression of its receptor CXCR4 and invasiveness of first trimester decidual HESCs (Ren et al., 2012). Trophoblast-secreted PDGFAA triggers decidualizing HESC chemotaxis (Schwenke et al., 2012). Gellersen et al. (2010) demonstrated that secretion of MMP2 and 9 was increased in HESCs upon decidualization. The authors also showed that decidualization bestows HESCs with increased migration and invasiveness, when co-cultured with an EVT cell line. These data indicate that, upon decidualization, HESCs acquire the ability to respond to embryo signals by increasing motility, migration and invasiveness. These properties enable encapsulation of the conceptus by the decidua (Quenby and Brosens, 2013, Weimar et al., 2013).

1.4.3.3 Control of Oxidative Stress Responses

In the first trimester of pregnancy, endovascular EVT form plugs in the spiral arteries, creating a hypoxic environment, which is essential to avoid generation of excessive amount of reactive oxygen species (ROS) (Burton et al., 1999). There are key roles for controlled oxidative stress in the development of the placenta, such as syncytialization, cytotrophoblast differentiation and angiogenesis (Myatt and Cui, 2004). Oxidative stress can lead to DNA repair (Tram et al., 2002), however it can also trigger cell cycle arrest (Barnouin et al, 2002), senescence (Toussaint et al., 2001), apoptosis (Kannan and Jain, 2000) and necrosis (Fiers et al., 1999), which could be deleterious for the vulnerable developing embryo. Oxidative damage at the maternal-foetal interface has been associated with early pregnancy loss (Jauniaux et

al., 2000), foetal growth restriction (Myatt and Cui, 2004) and preeclampsia (Burton and Jauniaux, 2004, Elliot, 2016).

There is evidence that decidualizing cells are adapted to inhibit excessive ROS signalling, preventing oxidative cell death, and maintaining pregnancy homeostasis (Gellersen et al., 2007). The expression of key antioxidants, such as glutathione peroxidase (GPX) 3, monoaminoxidase A and superoxide dismutase (SOD) 2 is up-regulated during the mid-secretory phase in the endometrium (Díaz-Gimeno et al., 2011). Uterine secretion of glutathione transferase and peroxiredoxin 4 is also increased in mid-secretory phase (Scotchie et al., 2009, Garrido-Gomez et al., 2010). Maruyama et al (1999) demonstrated increased secretion of the antioxidant thioredoxin by HESCs, upon treatment with E2 and P4. Xu et al. (2014) showed a reduction in pregnancy rate in mice, with intraperitoneal injection of a GPX inhibitor.

Growth arrest and DNA damage 45 α (GADD45 α) is a protein involved in response to stressful growth arrest conditions and DNA damage, acting as a gatekeeper to eliminate cells with excessive DNA damage. Up-regulation is observed in mid-secretory endometrium (Díaz-Gimeno et al., 2011) and in decidualized HESCs *in vitro* (Kajihara et al., 2006). This induction of GADD45 α is mediated by the cAMP-induced transcription factor FOXO1 (Tran et al., 2002).

Decidualizing HESCs are more resistant to oxidative cell death than un-differentiated cells. This effect is associated with the ability of differentiated HESCs to prevent up-regulation of FOXO α 3, upon oxidative stress (Kajihara et al., 2006).

Salker et al. (2011) studied the role of serum and glucocorticoid-regulated kinase 1 (SGK1) in implantation. SGK1 is a serine-threonine kinase induced in decidualizing HESCs. This enzyme plays an essential role in cellular stress response. mRNA levels were decreased and increased in mid-secretory endometrium from women suffering of recurrent pregnancy loss (RPL) and infertile women, respectively, when compared

with control subjects. Sgk1 knock-out mice exhibited decreased litter size. Upon *SGK1* knock-down, decidualizing HESCs showed a higher oxidation status in response to exogenous ROS (H_2O_2), and an impaired induction of free-radical scavengers (SOD2, thioredoxins, peroxiredoxin 2 and GPX1). Expression of the same scavengers was significantly lower in decidualizing HESCs from subjects with RPL compared to controls.

Taken together, these evidence point to a critical role of decidualization in bestowing a defence mechanism against excessive oxidative stress in the maternal-foetal interface.

1.4.3.4 Immune Tolerance to the Implanting Embryo

Pregnancy evokes an immunological conflict: the maternal organism must accommodate a non-self foetal semiallograft, providing a supportive environment, and safeguarding its own integrity. It is logical to question how the foetus elude maternal immune surveillance. However, evidence suggests that the decidual immune system has evolved to establish a cooperative arrangement with the trophoblast (Mor et al., 2011).

Decidualization bestows on the endometrial stromal compartment a specific immunological milieu, where specialized NK cells, dendritic cells (DCs), regulatory T cells (Treg cells) and macrophages interact to promote tolerance to the implanting blastocyst. In mice, the decidua prevents influx of cytotoxic T lymphocytes by silencing of chemokines through histone modification (Nancy et al., 2012).

DCs typically migrate to the local lymph nodes for antigen presentation. It was believed that, during pregnancy, these cells would be depleted in the maternal-foetal interface in order to avoid recognition of the semiallogenic embryo. Later, it was demonstrated that these cells, by changes of ECM and cytokine gradient, were indeed entrapped in the decidua (Collins et al., 2009, Erlebacher, 2013). DCs in

decidua secrete sFLT1 and TGF β 1, playing an important role in local angiogenesis. In mice, depletion of decidual DCs led to impaired implantation and embryo resorption (Plaks et al., 2008). Decidua macrophages contribute to reduced cytotoxicity of NK cells and cooperate with uNK cells to induce immunosuppressive Treg cells (Gormley et al., 2013, Vacca et al., 2010).

Galectin1 is involved in tumour immune evasion and restraint of autoimmune diseases, inhibiting T cell proliferation and survival (Camby et al., 2006). Galectin1-deficient mice exhibited increased foetal loss in allogenic, but not in syngeneic mating. Treatment with recombinant galectin1 prevented foetal loss and restored tolerance through induction of tolerogenic DCs and Treg cells (Blois et al., 2007). Kopcow et al. (2008) have demonstrated the apoptotic effect of uNK-derived galectin1 in activated T cells.

Decidua-derived Fas ligand triggers apoptosis of activated T cells in the maternal foetal interface and promotes trophoblast invasion (Qiu et al., 2005).

Indoleamine 2,3-dioxygenase (IDO), an enzyme highly expressed by decidualizing HESCs, degrades tryptophan, leading to growth arrest of T cells (Mellor and Munn, 2001). Pregnant mice treated with an IDO inhibitor exhibited extensive inflammation, massive complement deposition and haemorrhagic necrosis at foetal-maternal interface in allogenic, but not syngeneic pregnancies (Mellor et al., 2002).

uNK cells are less cytotoxic than their peripheral counterparts (Hanna et al., 2006). Local decidual factors contribute to the development of this special property. Co-culturing with decidualizing HESCs, and even conditioned medium from these cells, converted peripheral NK cells into an uNK cell-like phenotype (Keskin et al., 2007, Vacca et al., 2011). Cerdeira et al. (2013), using a combination of hypoxia, DNA demethylation and TGF β 1, managed to attenuate the cytotoxicity of peripheral NK cells, increase secretion of VEGF, and induce the ability of these cells to stimulate

trophoblast invasiveness. Recently, it has been demonstrated that decidualizing HESCs can reduce NK cytotoxicity, promote dendritic cell differentiation, and induce Treg cells (Erkers et al., 2013, Croxatto et al., 2014).

These data illustrate the compelling immunosuppressive potential of stromal decidualization, which may account for the immunological paradox of pregnancy.

1.4.3.5 Embryo selection

Reproduction in humans is exceptionally inefficient. The monthly fecundity rate is only 20 % (Evers, 2012). Human embryos are highly invasive, and chromosomal errors can be often found in preimplantation stages (Vaneste et al., 2009, Fragouli et al., 2013, Mertzaniidou et al., 2013). Considering pre-clinical losses, 50-60 % of pregnancies fail to proceed to an ongoing pregnancy, and more than 50 % of chromosome abnormalities are encountered in spontaneous miscarriages (Macklon et al., 2002). This indicates that numerous developmentally impaired embryos first implant to be eventually rejected and discarded. These data suggest that a selective mechanism operates, in order to limit maternal investment in a compromised conceptus. First evidence to propose the endometrium as a biosensor for embryo quality came from a study with livestock. Mansouri-Attia et al. (2009) demonstrated a tailored response of bovine endometrium to transferred embryos produced either by *in vivo* fertilization (artificial insemination; AI), somatic cell nuclear transfer (SCNT) or *in vitro* fertilization. The differences in peri-implantation endometrial gene expression were more pronounced between AI and SCNT embryos, with biological functions involving metabolism and immunity being identified.

Recent studies point to decidualization as the human process that bestow upon the endometrium the aptness to sense and select the embryos for support or exclusion. Teklenburg et al. (2010) showed that developmentally arrested embryos down-regulated the expression of implantation factors (IL1 β , IL6, IL10, IL, 17, IL18, eotaxin

and HB-EGF) in decidual HESCs, which was not observed with developing embryos. Furthermore, it was demonstrated that HESCs only engage in embryo sensing upon decidualization. The same group of researchers examined this role of the endometrium in the context of recurrent pregnancy loss (RPL). First, they demonstrated that endometrium and decidualized HESCs from patients with RPL expressed lower levels of *PRL* and higher levels of prokineticin 1 (*PROK1*), a factor induced during the receptive phase. Second, decidualized HESCs were treated with hCG, resulting in down-regulation of *PRL* and *PROK1* in the control group and up-regulation in the RPL group. Based on these data, the authors proposed that defective decidualization in RPL patients would elicit prolonged endometrial receptivity coupled with impaired selectivity (Salker et al., 2010). Brosens et al. (2014) tested the same hypothesis *in vivo*. Mouse uteri were flushed with conditioned media from developmentally impaired embryos (DIE) and embryos that resulted in ongoing pregnancy (developmentally competent embryos [DCE]), and were submitted to genome-wide expression profiling. While DCE triggered a modest response (15 differentially expressed genes [DEGs]), 449 DEGs were detected in DIE conditioned media-flushed uteri. Endoplasmic reticulum stress response was demonstrated to be induced by DIE. Decidualized HESCs were exposed to the same embryonic stimuli and displayed a similar response. It was also observed that cells treated with DCE conditioned media up-regulated genes involved in implantation and metabolism. Trypsin-like serine proteases were suggested as the putative embryo-released signal for the uterine luminal epithelium, relaying cues about embryo quality. A previous study showed that trypsin cleavage activated epithelial sodium channels in endometrial luminal cells from mice, triggering plasma membrane depolarization and Ca^{2+} influx. As a consequence, phosphorylation of the transcription factor CREB induced expression of the COX_2 gene (prostaglandin-endoperoxide Synthase 2 [PTGS2]), and eventually, PGE_2 secretion. This cytokine is a major ligand for activation of cAMP signalling (Ruan et al., 2012). *In vitro* trypsin evoked intracellular

Ca²⁺ oscillations in HEECs similar to those generated by DIE conditioned medium (Brosens et al., 2014). The oscillations induced by spent embryo medium were attenuated by a trypsin inhibitor. It was postulated that embryo-released serine proteases would signal to the luminal epithelium not only to modulate decidualization, but as well to allow embryo quality sensing.

Taken together these observations suggests that cyclic decidualization may limit maternal investment in invasive but developmentally impaired embryos.

1.5 Endometrial Glands

Uterine glands are part of the endometrial epithelial compartment, with ultrastructure and gene expression profile distinct from the luminal epithelium (Demir et al., 2002, Niklaus and Pollard, 2006). These structures are formed by a single layer of columnar cells in a tube-like arrangement that undergo constant modification along the menstrual cycle.

1.5.1 Development of Endometrial Glands

Uterine epithelium originates from the central tubular epithelium of paramesonephric ducts. All mammals share a similar sequence of events for gland development, termed adenogenesis. First, buds arise from luminal epithelium (LE), and next form a lumen as they progressively invaginate into the underlying stroma. In ruminants and primates, coiling and branching are further observed (Cooke et al., 2013). In rodents, pigs and sheep, gland morphogenesis is primarily a post-natal event (Cunha et al., 1976, Wiley et al., 1987, Bartol et al., 1993), whereas prenatal development of the uterus is crucial for future adenogenesis. In humans, fusion of the Müllerian ducts occurs before 8 weeks of pregnancy. By 20-22 weeks of gestation, sparse buds can be detected in the adluminal stroma. At birth, histoarchitecture is similar to that of an adult, though complete development, with glands extending to the myometrium, will

only take place in puberty (Gray et al., 2001). Curiously, these events are distinct from development in the post-menstrual phase and puerperium, where the glands arise from the crypts at the basalis and possibly from the stroma (Huang et al., 2012, Valentijn et al., 2013).

Adenogenesis is regulated in endocrine (steroid hormones), paracrine (factors expressed by stromal and LE cells), juxtacrine (cell-cell and cell-ECM matrix) and autocrine (factors expressed by glandular epithelium [GE]) manners (Figure 1.5). The cues control gland cell proliferation, differentiation, adhesion and motility, shaping the developing structures (Gray et al., 2001).

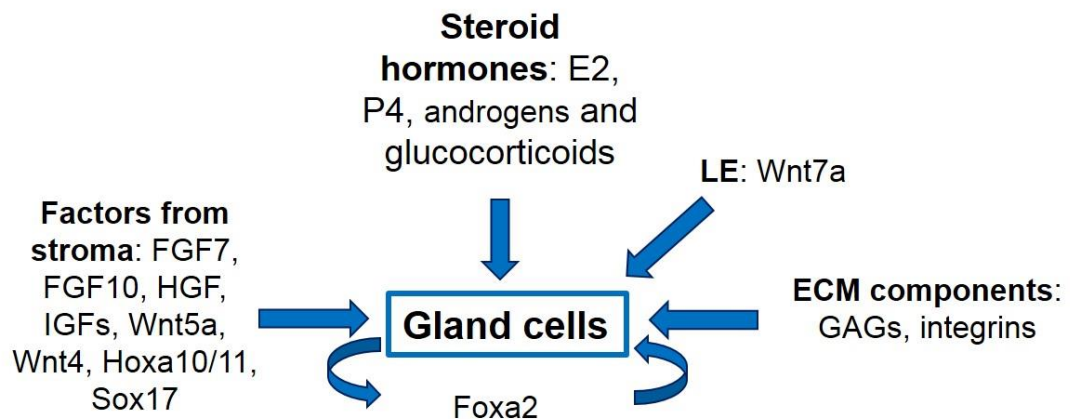


Figure 1.5 Control of endometrial adenogenesis. Uterine gland formation is regulated by steroid hormones, and factors derived from stroma, LE, ECM and GE.

The effect of steroid hormones in adenogenesis has been examined. P4 inhibits neonatal gland development, and this property has been explored to generate mice and female ewes devoid of uterine glands, allowing to unravel new roles for these secretory structures (Spencer et al., 1999, Cooke et al., 2012). The results with E2 are rather conflicting, varying between different species and the time of administration of the steroid (Branham, et al., 1985, Tarleton et al., 1999, Carpenter et al., 2003).

Branham et al. (1985) showed that exogenous E2 could alter adenogenesis in rats, in an age-specific manner. When administered between days 1 - 5 after birth, and between days 10 – 14, the final number of glands was reduced. Conversely, the use of E2 between days 20 – 24 increases the number of glands compared to controls. It was previously postulated that, despite the initial oestrogen-independent adenogenesis, ER α was required in the neonatal period, and was activated in a ligand-independent manner by growth factors such as EGF and IGF1 (Gray et al., 2001, Curtis et al., 1996, Klotz et al., 2002). However, this hypothesis was not supported by studies demonstrating that ablation of the ER α did not disturb gland formation (Stewart et al., 2011; Nanjappa et al., 2015). Simitsidellis et al. (2016) showed a striking increase of gland numbers in ovariectomized mice using dihydrotestosterone (DHT), revealing a novel role for androgens in endometrial function. Glucocorticoids have an antiproliferative effect on uterine epithelium, but the specific results in uterine glands have not been reported (Bigsby and Cunha, 1985). It has been proposed that at birth, the increased levels of both P4 from placenta and cortisol from parturition drop, ceasing the inhibition of uterine epithelial proliferation in female offspring (Cooke et al., 2013).

Epithelial-mesenchymal cross-talk can be mediated by modifications in the composition of ECM (Werb et al., 1996). Glycosaminoglycans (GAGs) can affect the cells directly or by enabling access of growth factors and other ligands to their receptors. During glandular development in other tissues (salivary glands, pancreas and mammary glands), sulfatated GAGs, e.g. chondroitin and heparans, are detected in morphogenically inactive areas, such as the neck of glands, whereas non-sulfated GAGs, e.g. hyaluronic acid, localizes in more active sites, such as the tips of the glands (Bernfield et al., 1973, Silberstein and Daniel, 1982). MMPs and their tissue inhibitors are also suggested to be involved in endometrial adenogenesis (Hu et al., 2004).

Wnt family members are secreted ligands that bind frizzled receptors (Fzd), a family of GPCRs, leading to activation of dishevelled proteins (Dsh). The activation of the canonical pathway promotes recruitment of the co-activator β -catenin (Ctnnb1) by target members of T cell factor/lymphoid enhancer factor (TCP/LEF) family to the enhancer elements of their target genes. These transcription factors regulate cell fate and morphogenesis (Nusse, 2012). Several Wnt members are involved in uterine adenogenesis in mice. Wnt4 and Wnt5a are secreted by the stroma and act on the gland cells of the epithelial compartment (Mericksay et al., 2004, Franco et al., 2011). By contrast, Wnt7a is produced by LE and acts on gland formation by controlling the expression of Wnt5a, Wnt4 and the morphogens Hoxa10 and Hoxa11 in the stroma (Dunlap et al., 2011). *Hoxa 10* mutant mice exhibits deficient endometrial gland patterning. Other members of the pathway, such as Wnt11, Wnt16 and Wnt7b, were localized in neonatal mouse uteri, although their ablation did not disturb the uterine phenotype. Fzd6 and Fzd10 were also found in LE, but studies about their role in adenogenesis are still lacking (Hayashi et al., 2011). Other evidence underpinning the importance of Wnt signalling is the impaired gland development upon ablation of *Ctnnb1*, *Lef1* and the co-receptor *Lgr4* (Jeong et al., 2009, Shelton et al., 2012, Sone et al., 2013). As mentioned previously, P4 inhibits adenogenesis if administered in the neonatal phase. Cooke et al. (2012) demonstrated inhibition of *Wnt7a*, *Wnt4*, *Hoxa10*, *Hoxa11* and *Fzd6* using P4 from postnatal day 3 until 9, suggesting a possible mechanism for the effect of this steroid. Diethylstilbestrol (DES), a synthetic oestrogen, was administered from day postnatal 1 to 5 and caused impaired adenogenesis and down-regulation of *Wnt7a* and *Wnt4* (Hayashi et al., 2011). The adhesion molecule e-cadherin (Cdh1) forms a complex with β -catenin and plays an important role in maintaining epithelial integrity (Tian et al., 2012). Ablation of *Cdh1*, in mice, resulted in disturbed endometrial adenogenesis and downregulation of *Wnt7a*, *Fzd6*, *Fzd10*, *Hoxa10* and *Hoxa11* (Benson et al., 1996).

The transcription factor forkhead box a 2 (*Foxa2*) is expressed exclusively by GE and its ablation disrupts uterine gland development in mouse (Jeong et al., 2010; Kelleher et al., 2017). Evidence indicates that *Foxa2* is regulated by β -catenin in the endometrium (Villacorte et al., 2013). A recent study has demonstrated that ablation of SRY (sex determining region Y)-Box 17 (*Sox17*) in endometrial stroma inhibited gland formation. Null mice exhibited decreased uterine expression of *Lef1*, and *Foxa2*, and increased expression of *Wnt7a*, although up-regulation was not observed at protein level (Guimarães-Young et al., 2016). *Dicer* encodes a ribonuclease involved in microRNA biosynthesis. MicroRNAs bind to complementary nucleotide sequences in untranslated regions of mRNA, leading to translation repression. *Dicer* knockout mice displayed reduced number of uterine glands on postnatal day 21, and presented aberrant expression of Wnt members, i.e. ectopic expression of *Wnt4* and *Wnt5a* in the epithelial compartment of endometrium (Gonzalez and Behringer, 2009).

Growth factors are also involved in endometrial gland morphogenesis. Stromal derived hepatocyte growth factor (HGF), and FGFs 7 and 10 are implicated in gland differentiation, proliferation and branching in other organs, such as lung and prostate (Bellusci et al., 1997, Ohmichi et al., 1998, Lu et al., 1999). HGF acts through binding to its receptor c-met, whereas FGF7 and FGF10 bind to a common epithelial receptor, defined as FGFR2IIIb (Komi-Kuramochi et al., 2005). Insulin-like growth factors 1 and 2 (IGF1 and IGF2) are secreted by stroma and, their biological effects are mainly mediated by the IGF1R, which is expressed in both endometrial compartments (Stevenson et al., 1994, Jones and Clemmons, 1995). Analysing neonatal ovine uteri, Taylor et al. (2001) have demonstrated an expression pattern of FGF7, FGF10, HGF, IGF1, IGF2, and their epithelial receptors in endometrial, remarkably consistent with the gland development. Furthermore, in another study, neonatal inhibition of

adenogenesis with a progestin, altered the expression pattern of these growth factors and receptors in neonatal ewe uteri (Gray et al., 2000).

Some evidence suggests a role for PRL in the development of uterine glands. PRL receptor (PRLR) is expressed in GE of sheep and humans (Jones et al., 1998, Taylor et al., 2000). Hyperprolactinemia induces endometrial gland hypertrophy (Kelly et al., 1997). Placental lactogen, another member of the PRL/growth hormone family induces uterine gland proliferation in sheep (Spencer et al., 1999).

1.5.2 Roles of Endometrial Glands

1.5.2.1 Histiotrophic Support of the Conceptus

The transition to viviparity requires the ability to support the conceptus whilst it remains within the maternal organism. Humans evolved to a haemochorial placenta, but during pre-implantation development, and until chorionic villi establish contact with maternal blood, the embryo must be nourished by decidual gland secretions (Burton et al., 2002). This mode of subsistence is named histiotrophic support.

Two circumstances led to the belief that the period of histiotrophic support would be rather short in human pregnancy. First, the invasive embryo is completely imbedded in the endometrial stroma by day 10 post conception, being sealed from the uterine luminal secretions (Gellersen et al., 2010). Second, histological examination of implantation sites suggests that migrating EVT, soon after implantation, invades maternal vasculature of the superficial endometrium (Burton et al., 1999).

Endometrial glands opening to the intervillous space (IVS) were documented in histologic sections of first trimester decidual-placental interface, and the same glycogen and lipid rich secretions observed in the glands were found dispersed in the IVS. Furthermore, glycogen staining was detected in the inner surface of villi, suggesting that products secreted by the decidual glands were absorbed by the

trophoblast. Immunostaining of utero-placental tissue for proteins specifically secreted by endometrial glands (glycodelin and MUC1), demonstrated staining of glands, IVS and villi close to the cytotrophoblasts shell. Glycodelin was further detected in villous macrophages, and MUC1 in small vesicles within the syncytiotrophoblast (Burton et al., 2002). Using co-staining with glycodelin and cathepsin D, a marker of lysosome activity, Hempstock et al. (2004) demonstrated digestion of the gland-derived protein in lysosomes within the chorionic villi of first trimester placentas. The authors also observed communications of gland lumens with IVS until at least 10 weeks. Moser et al. (2015) have even demonstrated the presence of endoglandular EVT as early as day 10 post-conception. These data indicate that the conceptus is not deprived from the uterine histiotrophe.

In vivo studies, using Doppler ultrasound, could only observe sparse sites with non-pulsatile flow in the intervillous spaces, before 10-12 weeks of gestation (Jaffe et al., 1997). Further histological studies of placenta-*in situ* specimens, with focus on the maternal-placental circulation, yielded some important observations. It was demonstrated that the spiral arteries are not invaded until 6 weeks, since they do not reach the superficial portions of the endometrium where the embryo is located. After 8 weeks, the connections between maternal arteries and placental villi were more frequent. However, aggregates of cytotrophoblast cells seems to plug the distal part of the arteries. Direct communications between endometrial arteries and IVS were only observed after 10 weeks of gestation (Burton et al., 1999). As result, a hypoxic environment is created in the first trimester of pregnancy. This hypothesis was confirmed by measuring oxygen tension within the placenta. A rise from < 20 mmHg at 8 weeks of pregnancy to > 50 mmHg at 12 weeks was concomitant with increased genomic expression and activity of antioxidant enzymes, such as catalases, GPXs and SODs (Jauniaux et al., 2000). Later, the same group analysed pH, O₂ and CO₂ tension of first trimester human foetal fluid (blood, amniotic fluid and exocoelomic fluid)

and utero-placental tissue, corroborating the previous findings (Jauniaux et al., 2001). The hypoxic environment coincides with the period when the organs are developing. The potential benefit of avoiding premature exposure to oxygen is to prevent excessive production of ROS, which could be deleterious for the fragile developing embryo. Free radicals could elicit DNA damage, disturbance of signalling pathways, nitrosylation of tyrosine kinases and mitochondrial stress-induced apoptosis, negatively affecting organogenesis (Ahmed and Rahman, 2015).

Considering that villi are poorly vascularised in the first weeks of pregnancy, the route by which nutrients are transported to the foetus is not obvious. Mesenchyme of early villi exhibit channels that blend with the mesenchyme lining the coelomic cavity at the chorionic plate (Castellucci et al., 1980). It is possible that molecules engulfed from the IVS diffuse through these channels into the coelom, and once there, they are absorbed by the well vascularized secondary yolk sac (Burton et al. 2001). In many species, vitelline circulation is the initial system used for gas and nutrient exchange (Carter, 2007). At the end of first trimester of gestation, when the haemochorial placenta is established, coelom is obliterated and secondary yolk sac degenerates (Burton et al. 2001). The high concentrations of folate and vitamin B12 in the coelom also suggest this surface is an important site for maternal-foetal exchange of nutrients (Campbell et al., 1993). Glycodelin was detected in the epithelium of the secondary yolk sac lining the coelomic cavity, at 8 weeks of gestation. At 12 weeks the staining was attenuated (Burton et al., 2002). These data suggest that the pathway through the coelom and secondary yolk sac may play a key role in nutrient exchange, before adequate villous vascularization is established.

The components of endometrial gland secretions responsible for embryo nutrition are not fully elucidated. The importance of these secretions for conceptus development inspired the term 'uterine milk' (Hansen et al. 1987). It is clear though, that glycogen, lipids, glycoproteins and micronutrients contribute to the nutrient milieu. Glycogen and

lipids were identified in human endometrial gland lumen by periodic acid Schiff and neutral red staining, respectively (Burton et al., 2002, Hempstock et al., 2004). The glycoproteins in the uterine milk lose the sialic acid capping during the pregnancy, facilitating absorption by the trophoblast and enhancing the availability of substrates for degradation. The latter role is desirable, for growth factors within IVS can occasionally enter maternal circulation via uterine veins, increasing the risk for cancer. This sialylation change may ensure early clearance of these factors by the liver (Burton et al., 2011). These modifications during pregnancy also indicate embryo modulation of uterine gland function. A recent study demonstrated how glycogen metabolism unfolds in decidual-placental interface. In the first trimester of pregnancy, glycogenolysis begins within the gland cells, by effect of glycogen phosphorylase, soon after synthesis by glycogen synthase. Further degradation occurs in the gland lumen via α -amylase. Part of the glycogen substrates are absorbed by syncytiotrophoblast, and used for metabolic processes; and part is reassembled and stored by cytotrophoblasts (Jones et al., 2015).

A high content of glycoproteins is also reported in uterine fluid (Burton et al., 2002, Hempstock et al., 2004; Jones et al., 2015). The most highly expressed of those is glycodeclin, also known as placental protein 14 (PP14), pregnancy-associated endometrial alpha-2 globulin (α_2 PEG) or progesterone-associated endometrial protein (PAEP). There are 3 isoforms of glycodeclin. Glycodeclin A is the endometrial isoform. This protein is involved in pregnancy immune tolerance, endometrial gland differentiation, blastocyst adhesion, and trophoblast invasion (Seppälä et al., 2002, Uchida et al., 2012). Its expression is mainly regulated by P4, but gland cells are also responsive to hCG (Seppälä et al., 1987, Fazleabas et al., 1999). Glycodeclin secretion increases until 10 weeks of gestation, and then declines (Seppälä et al., 1992). Another known glycoprotein secreted by uterine glands is MUC1. This anti-adhesion molecule has an important role in implantation, as previously mentioned and was

identified in uterine flushings (Hey et al., 1995). MUC1 expression in utero-placental interface can be strongly detected during first and second trimester of pregnancy, declining in the third trimester (Jeschke et al., 2002). Protein and lipids in uterine fluid have been assessed in an attempt to define a receptive profile (Beier and Beier-Hellwig, 1998, Scotchie et al., 2009, Salamonsen et al., 2013, Vilella et al., 2013). These studies identified a large number of secreted proteins and lipids, demonstrating a temporal change in the endometrial gland secretion composition, but the specific roles for each of these glandular secretion components are far from being elucidated.

1.5.2.2 Endometrial Receptivity and Embryo Implantation

A large body of evidence indicates that, apart from conceptus nourishment, the endometrial glands play a crucial role in endometrial receptivity and embryo implantation. Analysis of uterine luminal fluid (ULF) has identified cytokines, chemokines, proteases, protease inhibitors, several solute carriers, and other factors that modulates blastocyst and LE functions (Salamonsen et al., 2016). Once more, the progestin-induced gland knock-out animals helped to shed light on this uterine gland function. These animals were exposed to a progestin in the neonatal period, resulting in complete inhibition of adenogenesis. Uterine gland knock-out (UGKO) female sheep exhibited developed functional ovaries, did not present fertilization defects, and when mated with fertile males, normal blastocysts could be retrieved from uteri at gestation day 9 (Gray et al. 2001b). At day 14, conceptus were absent or severely growth-restricted (Gray et al. 2002). Progestin uterine gland knock-out (PUGKO) mouse phenotype includes infertility due to complete implantation failure. Intact embryos could be retrieved by flushing of uterine cavity on gestation day 5 (post-receptive phase), and those embryos implanted and resulted in normal pregnancy, when transferred to wild type recipients (Kelleher et al., 2016). PUGKO mice uteri displayed reduced uterine expression of key implantation genes (*Lif*, *Ihh*, *Prss28*, *Prss29* and *Hbegf*). In mice, clearance of the anti-adhesion protein MUC1

from LE, along with LE microvilli flattening and disappearance, a hallmark of polarity loss, are important parameters indicating endometrial receptivity. MUC1 remained abundant in PUGKO mice LE at day 4 (receptive phase). In addition, PUGKO uteri failed to undergo microvilli flattening and reduction in number (Kelleher et al., 2016).

Growth factors in ULF regulate conceptus proliferation, differentiation and adhesion. Hempstock et al. (2004) identified EGF, VEGF and TGF β expression in human GE. Receptors for these growth factors are present in trophectoderm of pre-implanting blastocysts. EGF receptors are expressed by trophoblast until 10 weeks of gestation, declining in later pregnancy (Ladines-Llave et al., 1991). EGF induces cytotrophoblast proliferation, and also stimulates hCG and human placental lactogen (hPL) secretion by syncytiotrophoblasts, in first trimester placental explants (Maruo et al., 1992). VEGF promotes trophoblast adhesion to LE and outgrowth of mouse embryos (Hannan et al., 2011, Binder et al., 2014). TGF β increases trophoblast fibronectin secretion, inducing adhesion (Feinberg et al., 1994). At the same time, this growth factor inhibits invasiveness, through down-regulation of vascular endothelial-cadherin protein (Chen et al., 2013). Furthermore, trophoblast stem cell proliferation is stimulated by TGF β in mice (Erlebacher et al., 2004).

GE also secretes other factors that promote trophectoderm adhesion to LE. Secreted phosphoprotein 1 (SPP1), also termed osteopontin, contains the integrin binding motif RGD, and mediates adhesion between embryo and LE (Singh and Aplin, 2009). This protein is induced in GE by P4, and promotes ovine trophectoderm cell attachment *in vitro* (Dunlap et al., 2008). LIF is an important protein secreted by GE, with multiple key roles in implantation, such as blastocyst/LE adhesion, embryo development, trophoblast differentiation and invasion, decidualization and leukocyte recruitment (Salleh and Geribabu, 2014). Recently, an interesting observation was the absence of LIF in mouse ULF (Kelleher et al., 2016). This finding suggests that this factor could be secreted basolaterally, rather than to the uterine lumen, acting on the LE and the

underlying stromal compartment. Glycodelin, a major secreted glycoprotein, as previously referred, is also involved in blastocyst interaction with uterine surface epithelium. Glycodelin induction in Ishikawa cells improves trophoblast spheroid attachment. This effect was completely abrogated by glycodelin gene (*PAEP*) silencing (Uchida et al., 2007). Proprotein convertase 5/6 (PC5/6) is a serine protease expressed by GE in mid-secretory phase. Cleavage of α -dystroglycan N-terminal in HEECs by PC5/6 is necessary for embryo attachment (Heng et al., 2015). Exosomes are 30-150 nm nanoparticles released from cells into the extracellular space, and used for intercellular communication. They were identified in human ULF, but some are retained in the glycocalyx (Ng et al., 2013). It has been suggested that the clearance of the glycocalyx during implantation releases exosomes, which are taken up by trophectoderm, switching it to a more adhesive phenotype (Salamonsen et al., 2015). Exosomes from P4 treated HEECs increased adhesive ability of trophoblast cells (Salamonsen et al., 2015).

Endometrial gland secretions in the mid-luteal phase also contain chemokines. CX3CL1, CCL14 AND CCL4 are amongst the most abundant attractants in human ULF, and their receptors are expressed by trophoblast cells. *In vivo* stimulation of trophoblast migration was demonstrated, and neutralizing antibodies to CX3CL1 and CCL4 attenuated this effect (Hannan et al., 2006). Transmembrane water channel, aquaporin 2 (AQP2) is mostly up-regulated during the mid-secretory phase, in GE and LE, at gene and protein level (He et al., 2006). This protein is involved in regulation of water absorption. In mice, during receptive phase, the volume of ULF decreases drastically, leading to 'lumen closure'. This phenomenon is essential for embryo apposition. In humans, a significant reduction in ULF has also been detected during the mid-secretory phase (Ruan et al., 2014). The control of fluid volume in the uterine cavity by AQP2 could contribute for the movement of the blastocyst to the eventual site of implantation.

Taken together, these data demonstrate the crucial role of endometrial glands in the establishment of uterine receptivity and the promotion of embryo implantation.

1.5.2.3 Stromal Decidualization

Histologic analysis of uteri of mouse models that are devoid of endometrial glands has demonstrated lack of decidualization upon pregnancy or pseudo-pregnancy (Jeong et al., 2010, Franco et al., 2011, Filant and Spencer, 2013a, Sone et al., 2013). Apart from the absence of uterine glands, these mice have a phenotype that resembles that of *Lif* null mice. LIF is solely expressed by GE in rodents, and is up-regulated during the receptive phase, in response to the nidatory E2 surge (Salleh and Giribabu, 2014). Conditional *Esr1* (*Era* gene) ablation in GE and LE leads to failed decidualization which is rescued by exogenous administration of LIF (Pawar et al., 2015). PUGKO mice are infertile due to implantation failure, and do not exhibit decidualization. These animals have normal oestrous cycle and expression of steroid receptors in the endometrium. Artificial decidualization in PUGKO mice after ovariectomy, using E2, P4 and intraluminal injection of oil, was attempted without success. The normal increase in uterine *Lif* expression in the receptive phase was abrogated (Filant and Spencer, 2013a). Similar results were observed with conditional ablation of *Foxa2*, *Wnt4* and *Lgr4* in mice. The phenotypes included failed adenogenesis and decreased *Lif* expression (Jeong et al., 2010, Franco et al., 2011, Sone et al. 2013). The results with LIF treatment, in replacement of E2 nidatory surge, were conflicting. Treatment could rescue decidualization defects in *Foxa2* and *Lgr4* ablation, but failed in PUGKO and *Wnt4* ablation mice. This suggests that there may be some other uterine gland-secreted factors involved in the process. Glands in receptive phase of pregnant and pseudopregnant mice express several candidate genes encoding for enzymes, solute carriers and secreted proteins that could be involved in the differentiation of the stromal cells (Filant et al., 2014, Filant and

Spencer, 2013b). Amongst these genes, serine peptidase inhibitor, Kazal type 3 (*Spink3*) is frequently present. *Spink3* mRNA is exclusively expressed in GE, whereas the protein is localized on both GE and LE in decidua. The enzyme is not regulated by the blastocyst, but is induced by P4 (Chen et al., 2010). *Spink3* is not conserved in human species, but its homolog, SPINK1, is a potent protease inhibitor, acting as an inactivation factor of intra-pancreatic trypsin activity. Mutations in the gene are associated with hereditary or chronic pancreatitis (Witt et al., 2000). Brosens et al. (2014) have suggested that SPINK1 could protect decidual cells from proteotoxic stress triggered by excessive activation of embryo proteases. A striking observation is the absence of LIF and *Spink3* in mouse ULF (Kelleher et al., 2016). It suggests that these factors are not apically released, but may be basolaterally secreted for signalling to the stroma.

In humans, maximal expression of LIF is observed during the mid-secretory phase in GE and LE (Paiva et al., 2009). LIF enhances P4-induced decidualization in HESC cultures, via STAT3 phosphorylation (Shuya et al., 2011). The cytokine shows an increase in human ULF in receptive phase (Laird et al., 1997) LIF could not be detected in supernatant of HESC, and concentration in HEEC supernatant was maximal when the cells were obtained from secretory phase endometrium (Laird et al., 1997). Besides LIF, many factors secreted by endometrial glands in women, such as IL11, TGF β , phospholipase A2 (PLA2), HB-EGF, KLF5 and IHH have been implicated in decidualization. IL11 and TGF β enhance P4-induced decidualization in HESC cultures (Dimitriadis et al. 2001, Kim et al., 2005). Phospholipase A2 releases arachidonic acid from membrane phospholipids (Rosenson and Gelb, 2009). Arachidonic acid is converted to prostaglandins by COX₂ (Greenhough et al. 2009). PGE₂, as previously mentioned, is a potent ligand for cAMP synthesis (Yee and Kennedy, 1991). Prostacyclin activates PPAR δ , leading to VEGF secretion. VEGF is involved in the angiogenesis process observed during decidualization (Prakasi and

Jain, 2008). The use of neutralizing antibodies, or an inhibitor for HB-EGF, attenuated cAMP-induced *PRL* expression in HESCs (Chobotova et al., 2005). Conditional KLF5 ablation prevents decidualization in mice. Furthermore, this factor was demonstrated to regulate *PTGS2* expression in HEEC (Sun et al., 2012). IHH is a mediator of PR signalling in the endometrium. Its effector COUP-TFII is also a regulator of stromal angiogenesis (Lee et al., 2006).

Even though the mechanisms are not fully elucidated, the evidence indicates an essential role for endometrial glands in stromal decidualization.

1.5.2.4 Immunomodulatory Effects of Endometrial Glands

Protection of the embryo from the maternal immune system is crucial for a successful pregnancy. Besides the aforementioned role of stromal decidualization, factors secreted by the endometrial glands also aid in this accommodation process.

Glycodelin A plays a key role in regulation of immune cell-rich decidual environment. Numerous immune suppressive effects have been described for this glycoprotein:

- Suppression of NK cell cytotoxicity (Okamoto et al., 1991)
- Inhibition of T cell proliferation/activity (Rachmilewitz et al., 1999)
- Induction of T cell apoptosis (SundarRaj et al., 2008)
- Induction of tolerogenic phenotype in dendritic cell (Scholz et al., 2008)
- Inhibition of B cell proliferation/activity (Yaniv et al., 2003)
- Inhibition of monocyte proliferation (Alok et al., 2009)
- Induction of monocyte apoptosis (Alok et al., 2009)
- Furthermore, glycodelin induces secretion of Th2 cytokines (IL6, IL13 and GM-CSF) from uNK cells, thereby favouring an advantageous Th2 dominant response in early pregnancy (Lee et al., 2011).

Other uterine gland products are also components of the innate immune system. Serpins are the most broadly distributed superfamily of protease inhibitors. All multicellular eukaryotes express serpins. In humans, some function as hormone transporters, molecular chaperones and tumour suppressors (Law et al., 2006). ULF of 16 day pregnant ewes contain SERPINA14, also called uterine serpin or uterine milk protein (UTMP; Koch et al., 2010). This serine protease inhibits lymphocyte proliferation, reduces humoral response against albumin, and inhibits NK activity (Padua and Hansen, 2010). SERPINA14 could inhibit miscarriage induced by polyinosinic:polycytidilic acid, a stimulator of NK activity, in mice (Liu and Hansen, 1993). This serpin is not expressed in humans, but *SERPING1* is up-regulated in mid-secretory phase endometrium in several genome wide profiling studies (Riesewijk et al., 2003, Mirkin et al., 2005, Talbi et al., 2006, Díaz-Gimeno et al., 2011). SERPING1 is a C1 esterase inhibitor, preventing spontaneous activation of the complement system (Law et al., 2006). Uteroglobins are cytokine-like anti-inflammatory proteins, produced by decidual glands. Uteroglobin is a potent inhibitor of neutrophil and monocyte chemotaxis *in vitro* (Kundu et al., 1996). Whey acidic proteins (WAP) are natural antimicrobials produced by mucosal surfaces (Bouchard et al., 2006). Secretory leukocyte protease inhibitor (SLPI) is a WAP, also known as antileukoproteinase 1. *SLPI* is also up-regulated in mid-secretory phase in endometrial transcriptome studies (Mirkin et al., 2005, Díaz-Gimeno et al., 2011, Hu et al., 2014), and the highest protein expression was found in first trimester decidua (King et al., 2000). Curiously, SLPI is also a member of the Kazal superfamily of serine protease inhibitors, just like SPINKs. Defensins (DEF) are small cationic proteins with antimicrobial properties. β -defensins (DEFB) are the main isoform secreted by epithelial glands. These proteins also function as immune cell chemoattractants (King et al., 2003). *DEFB1* is as well often detected as a mid-secretory up-regulated gene (Riesewijk et al., 2003, Talbi et al., 2006, Díaz-Gimeno et al., 2011).

These observations are consistent with a simultaneous role for endometrial glands in modulation of maternal immune tolerance to the embryo and protection of the intrauterine environment from pathogens.

1.5.3 Regulation of Endometrial Gland Secretion

Secretory activity of endometrial glands is regulated by the endometrial stroma (decidua), the embryo and the ovaries (CL).

Endocrine support of the decidualizing stroma is accomplished by P4 released from CL. This structure is, in turn, rescued from luteolysis by trophoblast derived chorionic gonadotropin. PRL is a major decidua-secreted protein. PRL receptor (PRLR) in pregnant ewe is exclusively localized in GE, and the expression of UTMP follows PRL production by the decidua (Stewart et al., 2000). In humans, PRLR shows maximal expression, at both transcript and protein level, in mid-secretory phase and first trimester GE (Jones et al., 1998). Jabbour et al. (2002) suggest that PRL activates JAK/STAT and mitogen-activated protein kinase (MAPK) pathways, promoting glandular differentiation. At the same time the activation of phosphatidylinositol 3 kinase (PI3K) pathway would prevent gland cell apoptosis. PRL also enhances P4-induced secretion of uteroglobin in mouse rabbit (Kleis-SanFrancisco et al., 1993). Besides inducing decidual secretion of stimulating factors, P4 also down-regulates PR expression in GE (Wang et al., 1998). First, it was believed that P4 directly promoted UTMP secretion by uterine glands, although this effect was only detectable after 14 days of treatment (Ing et al., 1989). Later it was demonstrated that down-regulation of PR was actually needed to prevent inhibitory effects of P4 on UTMP secretion (Spencer et al., 2004). The stimulatory action of P4 is most likely achieved, indirectly, by induction of decidual *PRL* (Burton et al., 2007).

Gland secretion diminishes in the late secretory phase of the menstrual cycle (McCluggage, 2011). By contrast, glycogen secretion increases until 6 weeks of

pregnancy (Burton et al., 2002). Besides CL-derived P4, factors released by the conceptus, seems to rescue the function of the endometrial glands. Indeed, embryonic signals are able to enhance uterine gland development and secretion in domestic animals (Spencer et al., 2004), and GE proliferation in early pregnancy has been demonstrated (Demir et al., 2002). Placental lactogen (PL) is a syncytiotrophoblast-secreted somatotropin that shares 67% sequence homology with PRL, and is able to bind and activate PRLR in GE (Burton et al., 2007). Intrauterine infusion of PL in ovine uteri increased the number of glands and induced expression of *UTMP* and *SPP1* mRNA in GE (Noel et al., 2003). Chorionic gonadotropin (CG) is another hormone secreted by syncytiotrophoblast and with a role in endometrial gland function. The maximal expression of the hCG receptor (LHCGR) is observed in LE and GE, during the secretory phase (Reshef et al., 1990). Human endometrial gland cells increased the expression of *PTGS2*, encoding COX_2 , and the secretion of PGE_2 upon exposure to hCG (Zhou et al., 1999). Female baboons exhibited up-regulation of glycodelin expression in the uterine glands, when treated with exogenous hCG (Hauserman et al., 1998).

A positive feedback mechanism is suggested between the endometrial glands and the conceptus. EGF secreted by the glands stimulates release of hPL and hCG by the syncytiotrophoblast. These peptide hormones induce secretory activity by GE (Burton et al., 2007).

In view of these observations, it can be conclude that endometrial gland function in pregnancy is regulated by coordinated roles of P4, the decidualized stroma and the conceptus (Figure 1.6).

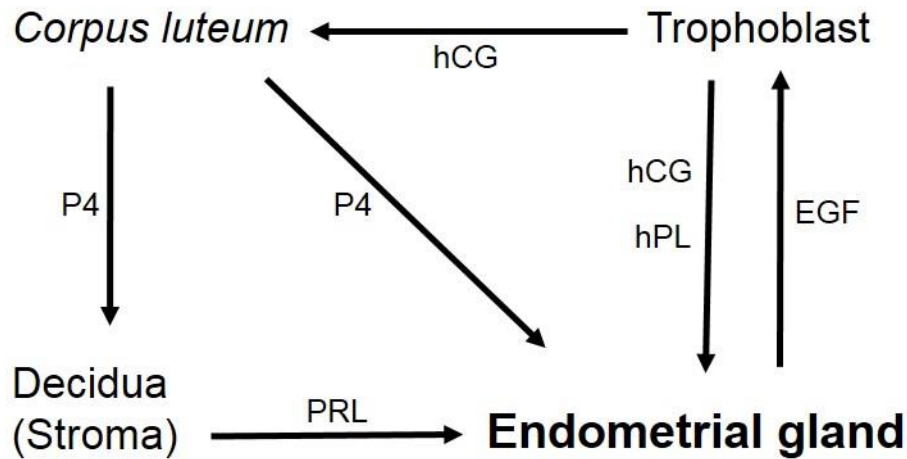


Figure 1.6 Regulation of endometrial gland secretion during pregnancy. Gland secretory activity, during pregnancy is regulated by the decidualized stroma, the corpus luteum, the embryo and by the endometrial glands themselves.

1.6 Missed Miscarriage

1.6.1 Spontaneous Miscarriage

According to the Royal College of Obstetricians and Gynaecologists, miscarriage is the spontaneous loss of pregnancy before foetal viability, meaning losses until 24 weeks of gestation. This concept tends to change over time, as survival of earlier babies is reported (Lawn et al., 2014). The terminology for the various types of miscarriage is controversial, with discrepancy and overlapping being found between the different nomenclature systems (Farquharson et al., 2005, Bottomley and Bourne, 2009, Kolte et al., 2014).

1.6.2 Epidemiology of Miscarriage

Approximately 15 % of all clinical pregnancies are lost, 98 – 99 % occurring within the first trimester (Regan and Rai, 2000). Risk increases with age. Miscarriage rate of 9% at 20 - 24 years old rises to 51 % in women aged 40-44 years (Andersen et al., 2000). β -subunit of hCG (β hCG) transcripts were identified as early as the two-cell stage of

human embryo development (Jurisicova et al., 1999). Extremely sensitive tests allow detection of β hCG in urine and serum 1 day after the implantation, engendering a new entity, the occult biochemical or pre-clinical pregnancy. In this scenario, an early positive β hCG test is not associated with clinical gestational symptoms, or even a positive ultrasound scan (Annan et al., 2013). Some of these women experience embryonic loss, before exhibiting an overt pregnancy. The real incidence of total miscarriages is difficult to estimate. Some women suffer the loss at home, without seeking for medical assistance. Others attend to different medical facilities. Furthermore, in many cases, the event presents too early to be distinguished from a normal menstrual bleeding or a slight delay in the menses. Nevertheless, when the pre-clinical losses are included, the incidence of total miscarriages can reach as high as 60% (Macklon et al., 2002).

Recurrent miscarriage is defined as 3 or more consecutive pregnancy losses before foetal viability. It affects 1% of couples, although the incidence rises to 1-5% if the definition adopted by many clinicians of 2 or more losses is considered (Rai and Regan, 2006).

1.6.3 Causes of Miscarriage

Numerous risk factors for miscarriage have been reported. However, establishing a real cause and the exact mechanism leading to the foetal loss, has been proven much more laborious. Several confounding factors may be involved, making it difficult to weigh the real contribution of a specific condition for the aetiology of the miscarriage. For example, polycystic ovary syndrome is frequently associated with insulin resistance, obesity and hyperprolactinemia (Bahceci et al., 2003). Some factors may be associated with recurrent miscarriage, whereas others can only justify sporadic cases. Infectious diseases, for instance, are usually associated with occasional, rather than repetitive pregnancy complications (Giakoumelou et al., 2015). Chromosome abnormalities are described as the major cause of miscarriage,

accounting for almost half of the cases (Goddijn and Leschot, 2000). Interestingly, the incidence of aneuploidies and structural aberrations in recurrent and sporadic miscarriages is the same (Stephenson et al., 2002, van der Berg et al., 2012).

Despite the plethora of putative pathologies associated with pregnancy loss, 40 - 50% of couples with recurrent miscarriage lack a diagnosis after the initial clinical investigation. In Table 1.3 some frequently described miscarriage risk factors are displayed with their possible causality.

Table 1.3 Risk factors for miscarriage and their causalities

Risk factor	Causality	Reference
Chromosome abnormalities	~45% of all losses. Similar incidence in recurrent pregnancy losses (RPL).	Stephenson et al., 2002 van der Berg et al., 2012
Parental balanced translocation	50% risk. Similar prognosis as other RPL causes recommends against PGD.	Treff et al., 2013 Franssen et al., 2006
Thrombophilias - Antiphospholipid syndrome (APS) - Deficiency of protein C - Deficiency of protein S - Factor V Leiden - Factor II mutation	Increased risk in APS. Not clear in congenital thrombophilias and no clear benefit for heparin and aspirin.	McNamee et al., 2012 de Jong et al., 2014
Endocrine - Hypothyroidism - PCOS - LPD	- Higher risk even with elevated autoantibodies and normal TSH. - Higher risk. No clear benefit with metformin in normal glucose tolerance. - Controversial diagnosis. Benefits with P4 for RM.	Marai et al., 2004 Zolghadri et al., 2008 Haas and Ramsey, 2014

<ul style="list-style-type: none"> - Hyperprolactinemia - Diabetes 	<ul style="list-style-type: none"> - Higher risk. No clear benefit with dopamine agonists. - Higher risk, only if not properly controlled. 	<p>Pluchino et al., 2014 Chen et al., 2016 Gutaj et al., 2013</p>
Infection	<p>Higher risk in malaria, brucellosis, CMV, HIV, dengue fever, influenza, bacterial vaginosis, rubella and syphilis.</p> <p>Not clear in Chlamydia, toxoplasmosis, HPV, HSV, parvovirus B19 and Hepatitis B</p>	Giakoumelou et al., 2015
Life style <ul style="list-style-type: none"> - Alcohol - Coffee - Smoking - Obesity 	<ul style="list-style-type: none"> - Higher risk - Increased risk with high doses - Higher risk - Higher risk 	<p>Andersen et al., 2012 Bech et al., 2005 Weng et al., 2008 Pineles et al., 2014 Talmor et al., 2015</p>
Uterine malformations	<p>Higher risk in septate, bicornuate and unicornuate uteri. Higher risk of second trimester loss with arcuate uterus. No clear benefit with metroplasty.</p>	<p>Chan et al., 2011 Kowalik et al., 2011</p>
Vitamins and oligoelements	<p>No association with a specific vitamin or oligoelement deficiency detected. Multivitamins and iron supplements reduce risk for stillbirth.</p>	Balogun et al., 2016
Natural killer cells	<p>No increased risk. Heterogeneity in methods used to measure number or density of the cells.</p>	Tang et al., 2011
Sperm DNA fragmentation	Higher risk.	Robinson et al., 2012

1.6.4 Missed Miscarriage

Missed miscarriage, also known as delayed miscarriage or silent miscarriage, is defined as a non-viable < 20 weeks pregnancy, and absence of uterine activity to expel the products of conception (Farquharson et al., 2005). Amongst some reported risk factors are folic acid deficiency, lack of physical exercise, hypoventilation and environment factors, such as ionizing radiation, pesticides, polycyclic aromatic hydrocarbons and phthalates (Zhang et al., 2011). A remarkable 13 - 24 fold increase in the incidence has been observed in China between 2002 and 2012, concomitant with escalating levels of industrial pollutants in the air (Yi et al., 2016).

The condition is found in 2.8 % of couples at 10-14 week pregnancy (Pandya et al., 1996). Besides the unexpected ultrasound finding in asymptomatic patients, there are cases of women with a mild first trimester bleeding and a long closed cervix, or the report of bad premonition, after the ceasing of pregnancy symptoms (Jansson and Adolfsson, 2010). The final diagnosis is usually achieved by serial ultrasound scans and common echographic signs include:

- Persistent empty gestational sac (GS) (Kolte et al., 2015).
- Small GS for pregnancy age calculated by the last menstrual period (LMP), also called early oligohydramnios (Odeh et al., 2010).
- Absence of foetal heart activity after 6 weeks of pregnancy or crown-rump length (CRL) ≥ 5 mm (Farquharson et al., 2005).
- Failure of CRL to progress in one week (Bottomley and Bourne, 2009).
- CRL small for gestational age, calculated by LMP (Farquharson et al., 2005).
- Foetal bradycardia (Makrydimas et al., 2003).
- Abnormalities in yolk sac: large, small, unusual shape, calcification, or early regression (Cho et al., 2006).

For the purpose of this thesis missed miscarriage was considered as first trimester intrauterine visualized losses and empty sacs. Ectopic pregnancies and pregnancies of undetermined location were also excluded.

1.6.5 Etiopathogenesis of Missed Miscarriage

There are no defined specific conditions associated with missed miscarriages, but some mechanisms have been suggested. Several studies addressing this type of foetal loss direct the main focus on the discovery of diagnostic markers, thereby not being possible to discriminate whether the finding is rather a cause than a consequence of the pregnancy failure.

There is no precise estimate for the incidence of chromosome abnormalities in missed miscarriage cases. Nevertheless, some studies coupling embryoscopy with karyotyping suggest a higher frequency when compared to general miscarriages. Philipp and Kalousek (2001) found 9 (60 %) abnormal karyotypes in 15 embryos. In a larger series, the same group demonstrated 70 % of chromosome errors in 37 cases of missed miscarriage (Philipp et al., 2002). Ferro et al (2003), after analysing 55 karyotypes of direct embryo biopsies, detected 37 (67.3 %) chromosome abnormalities. An even higher incidence of abnormal karyotypes was observed again by Philipp et al. (2003). These authors identified 165 (75 %) chromosome errors amongst 221 missed miscarriage cases. A remarkable finding in all of these studies was the frequency of morphologically abnormal embryos with a normal karyotype that varied from 18 % to 35.7 %. In another study, 177 (65 %) abnormal karyotypes were encountered in 272 curettages of missed miscarriages, 58 % amongst empty sacs and 68 % amongst retained embryos (Lathi et al., 2007). Askerov (2016), investigating 59 cases of anembryonic pregnancies, detected 71.4 % of chromosome abnormalities in spontaneous pregnancies, whereas between the pregnancies after assisted reproduction techniques, the incidence was 55.3 %. However, even

considering the high incidence of chromosome errors, 25–40 % of missed miscarriage cases may occur due to other aetiologies.

Angiogenesis is crucial for the development of functional chorionic villi. The hypoxic environment of the maternal-placental interface in the first trimester induces the expression of several angiogenic factors and their inhibitors (Charnock-Jones et al., 2004). Impaired angiogenesis has been implicated in the genesis of missed miscarriages. Reduced serum levels of the angiogenic placental growth factor (PlGF) and the VEGF inhibitor serum fms-like tyrosine kinase 1 (sFlt1) were associated with missed miscarriage, and were postulated to occur as a consequence of decreased local hypoxia (Daponte et al., 2011, Martinez-Ruiz et al., 2014). Precocious increase in oxygen levels in IVS, would disturb placental angiogenesis. Lower serum levels of angiopoietin 1 and 2 were also verified in delayed miscarriage cases (Daponte et al., 2013a). The main issue with these investigations on angiogenesis is the focus on the discovery of markers to discriminate between normal pregnancy and missed miscarriage or even between missed miscarriage and ectopic pregnancy. Fang et al. (2013) demonstrated dysregulation of the hypoxia induced factor 1 α (HIF1 α)/VEGF/sFlt1/delta-like ligand 4 (Dll4)/Notch 1 pathway in placental villi of missed miscarriages. The authors suggested that excessive hypoxia would disarrange this system, resulting in reduced expression of the Notch 1 ligand, Dll4, an essential factor for establishment of vascular endothelial cell fate.

Placental apoptosis has essential roles in early pregnancy, ensuring maternal immune tolerance to the implanting embryo, and steering trophoblast remodelling (Jersak and Bischof, 2002). However, excessive villous apoptosis may increase the risk for early pregnancy complications (Rull et al., 2013). Cells from amniotic and coelom fluid acquired from missed miscarriages exhibited higher expression of FAS and FAS ligand. These are main factors that control apoptosis. Furthermore, the same samples and trophoblast samples from the same patients harboured a higher

number of cells stained positive for terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) (Kaponis et al., 2008). TUNEL is a method for detecting apoptotic cells that undergo extensive DNA degradation (Kyrylkova et al., 2012). Gene associated with retinoid-interferon mortality 19 (*GRIM19*) is an anti-apoptotic gene essential for the assembly of mitochondrial complex 1 (Huang et al., 2004). Expression of GRIM19 was downregulated in trophoblast from missed miscarriage patients. The expression of P53 was up-regulated and the number of TUNEL+ cells was higher when compared with normal pregnancies. In addition, the expression of HIF1 α was increased, and that of VEGF was reduced (Chen et al., 2015). P53 is a pivotal mediator of apoptosis in response to DNA damage. Human double minute 2 (*HDM2*) is induced by P53 and is a key inhibitor of P53 activity. A specific combination of gene polymorphism for *P53* and *HDM2* was observed in association with recurrent miscarriage (Fang et al., 2011). The authors suggested that this gene-gene polymorphism interaction would blunt the apoptotic process in the placenta. Thereby the clearance of DNA damaged cells would be impaired, resulting in foetal demise. These data underscores the importance of a fine balanced control of apoptosis in the human placenta.

Decidua- and placenta-derived cytokines are critical to ensure maternal immune tolerance to the foetus and a successful pregnancy (Mor et al., 2011). Imbalance in the secretion of the various members can lead to pregnancy disorders (Raghupathy and Kalinka, 2008). A shift from an anti-inflammatory Th2-type cytokine response (i.e. secretion of IL4 and IL10) to a pro-inflammatory Th1-type cytokine response (i.e. secretion of interferon γ [IFN γ], IL2, sIL2R, IL12, IL15, TNF α) has been indicated as a possible cause for missed miscarriage. Plevyak et al., (2002) observed decreased expression of IL10 and no changes in IFN γ and immune cells in decidua from missed miscarriage patients. Daponte et al., (2013b) detected increased serum levels of IL15 and no changes in anti-C1q in patients with delayed early pregnant loss. Increased

serum levels of IL12 and sIL2R were also described in cases of missed miscarriage, when compared to normal pregnancy and even threatened miscarriage (Paradisi et al., 2003). In a more recent study, increased serum levels of IFN γ and reduced IL4 and peripheral Treg cells were verified in missed miscarriage cases (Cao et al., 2014). Based on the additional observation of decreased E2 serum concentrations, the authors suggested a positive correlation of the low levels of T reg cells and IL4 with the low concentration of that sex steroid.

Metabolomics is the study of endogenous low-molecular-weight metabolites that could serve as disease-specific biomarkers, or provide insights into mechanisms of a disorder (Zhang et al., 2012). In a recent paper, this approach was used in an attempt to define biomarkers for missed miscarriage (Fei et al., 2016). Thirty-seven differential plasma metabolites that discriminate between normal pregnancies and missed miscarriages were detected. Using metabolic impact analysis, two metabolic pathways were identified: tryptophan and sphingolipid metabolism. Lower level of tryptophan and their metabolites were observed (Fei et al., 2016). Tryptophan degradation is essential for maternal immune tolerance to the foetus via growth arrest of T cells (Mellor and Munn, 2001). This amino-acid is also crucial for foetal growth and development during the organogenesis period (Badawy, 2015). It is also a precursor of serotonin, and placenta-derived serotonin is involved in foetal brain development (St-Pierre et al., 2016). As regards the sphingolipid metabolism, higher plasma levels of sphingosine and reduced levels of sphingosine 1-phosphate (S1P) were detected in cases of missed miscarriage (Fei et al., 2016). Sphingosine inhibits proliferation and induces apoptosis and inflammation, whereas S1P has anti-apoptotic, anti-inflammatory and angiogenic properties (Gomez-Muñoz et al., 2016). Sphingolipid metabolism pathway is also critical for prevention of foetal rejection by regulation of innate immunity at the feto-maternal interface (Mizugishi et al., 2015). In

addition, S1P was demonstrated to play a role in the maintenance of the corpus luteum in mice (Hernandez et al., 2009).

Oxidative stress has been implicated in RPL (Gupta et al., 2007). Elevated serum levels of lipid peroxidation and reduced levels of reduced glutathione, GPX, vitamin A, vitamin E and β -carotene have been observed in patients who suffered recurrent miscarriages (Şimşek et al., 1998). Decreased plasmatic levels of the antioxidants α -tocopherol, vitamin C and erythrocyte glutathione and total thiols were also associated with repeated miscarriages (Vural et al., 2000). Sata et al. (2003) reported increased risk for RPL in glutathione S-transferase M1 (*GSTM1*) and T1 (*GSTT1*) gene polymorphism carriers. These enzymes are important free radical scavengers (Hayes and Strange, 2000). Based on the findings of Nelen et al. (2000) associating homocysteinemia and RPL, Gupta et al. (2007) postulated that the pro-oxidant property of homocysteine would be the mechanism responsible for the pregnancy failures. Up-regulated expression of the oxidative stress markers hsp 70 and nitrotyrosine, alongside with increased lipid peroxidation in placental specimens were described in missed miscarriage (Hempstock et al., 2003). Sugino et al. (2000), compared oxidative stress in decidua from normal early pregnancies, missed miscarriages and spontaneous miscarriages with vaginal bleeding. Total SOD activity, Cu,Zn-SOD activity and mRNA were decreased, and lipid peroxidase and PGF2 α were increased in spontaneous miscarriage with bleeding. By contrast, there were no differences between normal pregnancies and retained conceptuses. The authors proposed that the elevated ROS levels would induce PGF2 α synthesis in miscarriages with spontaneous expulsion of conceptus. This phenomenon would be abrogated in missed miscarriages. However, in the study it was not possible to ascertain if the stress response was a cause or a consequence of the increased uterine activity. More recently, a study showed a decrease in SOD and HIF1 α expression, and increased ROS levels in trophoblasts from missed miscarriage

patients, indicating a possible mechanism for foetal demise through disturbed angiogenesis (Zhu et al., 2014).

Although most knowledge on this type of pregnancy loss is derived from serum, placenta and, in a lesser proportion, decidua analysis, no investigation on endometrial glands has been hitherto performed. Furthermore, as previously mentioned, all these studies have been performed during the miscarriage time-frame, thereby not being possible to determine if the conditions were cause or consequence. Considering the crucial role of uterine glands in providing nutrients and growth factors for the embryo in the early pregnancy, it is rational to presume that glandular defects could lead not only to inadequate foetal nutrition, but also to impaired organogenesis and eventually foetal death. The result would be a malformed foetus or an empty sac with a normal karyotype. Indeed several papers have described structurally abnormal foetuses at embryoscopy as well as empty sacs with normal karyotypes (Phillipp and Kalousek, 2002, Ferro et al., 2003, Lathi et al., 2007).

1.7 Research justification and aims

The role of endometrial glands in the implantation process and early pregnancy remains poorly understood. A major hurdle is the inability to successfully propagate and differentiate primary HEECs in culture, which contributes to the paucity of studies focusing on glands in the endometrial field. Hence, the overall aim of my project is to develop efficient methods for isolation, culturing and differentiation of primary HEECs *in vitro*. The specific goals are:

- To establish 2D and 3D models to study of primary HEECS and human endometrial glands in culture, respectively.
- To study the effect of ovarian steroids, embryo-derived signals and stromal decidual cues on HEEC cultures.
- To investigate the role of uterine glands in the pathophysiology of recurrent missed miscarriages.

Chapter 2

Materials and methods

2.1 Materials and Recipes

2.1.1 List of Materials

Table 2.1 Materials used in the experiments, supplier and catalog numbers.

Material	Supplier	Catalog number
β -actin antibody Mouse	Abcam	AB8224
β -estradiol	Sigma-Aldrich	E2758
β -mercaptoethanol	Sigma-Aldrich	M3148
3T3 swiss albino mouse embryo fibroblasts (cell line)	Sigma-Aldrich	85022108
8-Bromoadenosine 3',5'-cAMP	Sigma-Aldrich	B7880
6x DNA gel loading dye	Thermo Scientific	R0611
6 well plates	Corning	3598
12 well plates	Corning	3513
24 well plates	Corning	3506
96 well plates	Corning	3599
Acrlamide, 30%	Bio-Rad Laboratories	161-0158
Adenine -	Sigma-Aldrich	A2786
Advanced DMEM/F12	Life Technologies	12634010
ALK-4, -5, -7 inhibitor, A83-01	Sigma-Aldrich	SML0788
Ammonium persulfate (APS)	Sigma-Aldrich	A3678

Antibiotic/antimycotic (Penicilin, streptomycin, amphotericin B)	Gibco	15240-062
B27 supplement minus vitamin A	Life Technologies	12587010
Black 96-well plate for fluorescence	Appleton Woods	611F96BK
BSA	–Sigma-Aldrich	A2153
Cell Recovery Solution	Corning	354253
Cell scrapers	Corning	3010
Cell strainer, 40 µm	Fisher Scientific	22363547
Charcoal Sigma-Aldrich	Sigma-Aldrich	C9157
Chloroform	VWR	100034Q
Cholera toxin	Sigma-Aldrich	C8052
Cleaning agent for removing RNase (RNAzap)	Sigma-Aldrich	R2020
Collagenase	Sigma-Aldrich	C9891
Cytokeratin 18 antibody Mouse	Abcam	AB668
DNase I for tissue digestion	Roche	11284932001
Dextran 70	Sigma-Aldrich	1179741
DL-dithiothreitol (DTT)	Sigma-Aldrich	D9779
DPX mounting medium	Leica	3808600E
Dulbecco's modified Eagle medium (DMEM)/F12	Gibco	31330-638

nutrient (Ham) (1:1) medium with red-phenol		
Dulbecco's modified Eagle medium (DMEM)/F12 nutrient (Ham) (1:1) medium with red-phenol	Gibco	11039-021
E-cadherin antibody Rabbit	Cell Signaling	3195
ECL Western-blotting analysis system	GE Healthcare	RPN2232
ENaC α antibody Sigma	Sigma-Aldrich	HPA012743
ENaC β antibody	Sigma-Aldrich	HPA015612
ENaC γ antibody	Proteintech	13943-1-AP
Eosin Y	Sigma-Aldrich	HT110132
Epidermal growth factor (EGF)	Peprtech	AF100-15
Eppendorf tubes 1.5 ml	Starlab	E1415-1500
Eppendorf tubes 0.6 ml	Starlab	E1405-0600
Ethanol	Fisher Chemical	E/0650DF/17
Falcon tubes 14 ml	Greiner Bio-one	188261
Falcon tubes 50 ml	Greiner Bio-one	227270
Fetal bovine serum (FBS)	Gibco	10500-64
FGF10	Peprtech	100-26
Filter paper	Whatman	3001-861
Formaldehyde, 4%	VWR	9713

Frostbite freezing spray	Leica	3803100EE
Gel cassettes	Novex	NC2015
Glass-bottom Petri-dishes	Mat tek	P35GCol-1.5-10-C
Glass Pasteur pipettes	Fisher Scientific	1156-6963
Glass slides	Thermo Scientific	BS7011/2
Gloves	Kimtech Science	90626
Glycogen	Invitrogen	10814-010
Hanging Drop plates	Sigma Aldrich	HDP1096
hepatocyte growth factor (HGF)	Peprotech	100-39
Human chorionic gonadotropin (hCG)	Sigma-Aldrich	C0434
Histone H3-MeK9 antibody Rabbit	Abcam	AB8898
HMGB2 antibody Rabbit	Abcam	AB672282
Hydrocortisone	Sigma-Aldrich	H0888
Hyper ladder V	Bioline	BIO-33031
Insulin	Sigma-Aldrich	91077C
Isopropanol (2-Propanol)	Sigma-Aldrich	24137
Isolation caps with diffuser for LMD	Molecular Machines Industries	50202
Lamin B1 antibody Mouse	Abcam	AB8982
LDS buffer NuPAGE	Invitrogen	NP0008

L-glutamine	Gibco	25030-024
Matrigel	Corning	354230
Medroxiprogesterone 17-acetate	Sigma-Aldrich	M1629
Membrane slides	Molecular Machines Industries	50103
Methanol	Fisher Chemical	M/4000/17
Microtome blade	Leica	R-35
N2 supplement	Life Technologies	17502048
N-acetyl-L-cysteine	Sigma-Aldrich	A9165
Nicotinamide	Sigma-Aldrich	N0636
N, N, N',N'-tetramethylethylenediamine (TEMED)	Sigma-Aldrich	T9281
Noggin	Peprtech	120-10C
Nonfat dried milk powder	Panreac AppliChem	A0830
Nuclease-free water	Ambion	AM9932
OCT cryoembedding matrix	VWR	36160E
P16 antibody Rabbit	Abcam	AB108349
P53 antibody Mouse	Dako	M7001
Parafilm	Bemis	PM-996
PBS	Fisher Scientific	102092521X-PBS
PCR plates FAST	Applied Biosystems	4346906

PCR plates non-FAST	Applied Biosystems	N8010560
PCR plate sealing film	Excel Scientific	TS-RT2-100
Petri-dish 92 x 16 mm	Sarstedt	821473
Pipette tips 10 µl	Alpha Laboratories	ZP1010S
Pipette tips 40 µl	Alpha Laboratories	ZP1204S
Pipette tips 100 µl	Alpha Laboratories	ZP1200S
Pipette tips 300 µl	Alpha Laboratories	ZS3300S
Polyvinylidene Fluoride (PVDF) blotting membranes	GE Healthcare	10600023
Prestained protein ladder	Thermo Scientific	26619
Progesterone	Sigma-Aldrich	P7556
Prolactin	Peprtech	100-07
Protease inhibitor	Roche	11836170001
Protein assay dye reagent	Bio-rad	5000006
RTCA plates (E-plate)	ACEA Biosciences	05469813001
R-spondin 1	Peprtech	120-38
RIPA buffer	Upstate Cell Signalling Solutions	20-188
Scalpels	Swann-Morton	0501
Secondary antibodies HRP anti-mouse	Dako	P0447
Secondary antibodies HRP anti-rabbit	Dako	P0448

Secondary antibody, anti-mouse alexa-fluor 488	Molecular Probes	A11088
Secondary antibody, anti-mouse alexa-fluor	Molecular Probes	A21424
Serological pipettes 5 ml	Greiner Bio-one	606 180
Serological pipettes 10 ml	Greiner Bio-one	607 180
Serological pipettes 25 ml	Greiner Bio-one	760 180
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich	75746
Sponge pad for blotting	Novex	E19052
STAT-60	AMS Biotechnology	CS-502
Sybr green	Applied Biosystems	4367659
Syringes 20 ml	BD Plastipak	300613
Syringes 50 ml	BD Plastipak	300865
Syringe filter	Sartorius Stedim Biotech	16534-K
Transwell cell culture inserts	BD Falcon	353095
Triton X-100	Sigma-Aldrich	T9284
Trypsin	Sigma-Aldrich	T4799
Trypsin-EDTA 0.25%	Gibco	25200-056
Tween 20	Fisher Scientific	EC500-018.3
Vectashield mounting medium with DAPI	Vector Laboratories	H-1500
Vimentin antibody Mouse	Cell Signalling	3390

Y-27632	Abcam	AB120129
Xylene	Fisher Chemicals	X/250/17
Gel extraction/purification of DNA	Qiagen	28704
Glycogen assay Kit	Abcam	AB169558
Immunohistochemistry	Leica	RE7150-K
Laser microdissection staining kit	Ambion	AM1935
On-column DNase I digestion set	Omega Bio-Tek	E1091
PGE ₂ ELISA kit	Thermo Scientific	EHPGE2
Reverse transcription kit	Qiagen	205314
RNAqueous Micro Kit	Ambion	AM1931
Senescence-associated beta galactosidase activity kit (fluorometric format)	Cell Biolabs	CBA-231
Senescence-associated beta galactosidase staining kit	Cell Signaling	98605

2.1.2 Recipes

2.1.2.1 Blocking Buffer for Blotting

5% Skimmed milk powder (w/v) in TBS-T

2.1.2.2 Blocking Buffer for Immunofluorescence:

1% BSA (w/v) in PBS

2.1.2.3 Running Buffer (10 X)

250 mM Tris base

192 mM Glycine

1% (w/v) SDS

2.1.2.4 Transfer Buffer (10 X)

250 mM Tris base

192 mM Glycine

20 % (v/v) Methanol

2.1.2.5 DNA Loading Buffer

0.3 % (w/v) Bromophenol blue

40% (v/v) Glycerol

0.25 M EDTA, pH 8.0

2.1.2.6 Tris-Borate EDTA (TBE) Buffer (10 X)

0.9 % Tris-borate

2 mM EDTA, pH 8.0

2.1.2.7 Tris-Buffered Saline (TBS) (10 X)

130 mM NaCl

20 mM Tris

Adjust pH to 7.8

2.1.2.8 TBS-T

0.1 % Tween 20 in 1X TBS

2.1.2.9 Tris-EDTA Elution (TE) Buffer

10 mM Tris-HCl pH 8.0

1 mM EDTA

2.1.2.10 Citrate Buffer

Sodium citrate 10 mM

0.05 % Tween 20

2.1.2.11 Whole Cell Extraction Buffer (WEB buffer)

20 mM Hepes 0.5% NP-40

0.5 % NP-40

15 % glycerol

0.3 M NaCl

0.5M EDTA

1 mM Na_3VO_4

10 mM NaF

1 mM DTT

2 mM leupetin

1.5 mM pepstain

150 μM aprotinin

0.6 mM phenylmethylsulfonyl fluoride (PMSF)

2.1.2.12 10% dextran-coated charcoal (DCC) Medium

500 ml DMEM/F12 with phenol red

50 ml DCC

2 mM L-Glutamine

5 ml Antibiotic-Antimycotic solution (100 X) 5 ml

2 µg/ml Insulin

1 nM Oestradiol

2.1.2.12 Conditioned Reprogramming of Epithelial Cell Medium (CRC Medium)

500 ml DMEM/F12 with phenol red

50 ml DCC

2 mM L-Glutamine

5 µg/ml Insulin

5 ml Antibiotic-Antimycotic solution (100 X) 5 ml

8.4 ng/ml Cholera toxin

10 ng/ml EGF

0.4 µg/ml hydrocortisone

24 µg/ml Adenine

10 µM/I Y-27632

2.1.2.13 Organoid Expansion Medium

500 ml Advanced DMEM/F12

5 ml N2 supplement (100 X)

10 ml B27 supplement minus vitamin A (50 X)

5 ml Antibiotic-antimycotic solution (100 X) 5 ml

1.25 mM N-acetyl-L-cysteine

2 mM L-glutamine

50 ng/ml EGF

100 ng/ml Noggin

500 ng/ml R spondin-1

100 ng/ml FGF10

50 ng/ml HGF

500 nM ALK-4, -5, -7 inhibitor

10 mM Nicotinamide

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Procurement of Endometrial Tissue

Endometrial tissue was acquired from women attending the Implantation Clinic at University Hospitals Coventry and Warwickshire National Health Service Trust, after obtaining written consent. The study was approved by the NHS National Research Ethics Committee of Hammersmith and Queen Charlotte's Hospital NHS Trust. Endometrial biopsies were collected under ultrasound guidance, using a Wallach Endocell sampler (Wallach Surgical Devices, Trumbull, CT, USA), and were timed to the mid-secretory phase of the menstrual cycle. LH surge was self-assessed by the patients through a home urine ovulation test. None of the women were using hormones for at least three months prior to the tissue collection. A fragment of the tissue was put in 10 % DCC medium for immediate isolation of endometrial cells. Another fragment was immediately snap frozen in liquid nitrogen and kept at - 80 °C until its use for laser microdissection.

2.2.1.2 Preparation of Dextran-Coated Charcoal Treated Stripped Foetal Bovine Serum (DCC)

Foetal bovine serum (FBS) was stripped of several small molecules, including endogenous hormones, by treatment with dextran-coated charcoal. Here, 1.25 g charcoal and 125 mg dextran were added to 500 ml FBS and incubated at 56 °C for 2 hours with intermittent shaking. The mixture was centrifuged at 1100 g for 30 minutes, before the supernatant was filtered to remove the charcoal, and aliquoted and stored at - 20 °C.

2.2.1.3 Isolation of Endometrial Stromal Cells (HESC)

The endometrial tissue was minced for 5 minutes with a scalpel, and digested in an enzymatic solution (composition: 0.1 mg/ml DNase I and 0.5 mg/ml collagenase in 10 ml phenol red-free DMEM/F12) for one hour at 37 °C with intermittent shaking. After 1 hour, collagenase was neutralized by addition of 10 ml 10 % DCC medium and centrifugation at 280 g for 5 minutes. The cell pellet was re-suspended in 10 % DCC medium and seeded into tissue culture flasks. Blood and epithelial cells in suspension were removed by changing media after 3 hours and within 18 hours. Media were changed every other day until the cells were confluent.

2.2.1.4 Isolation of Human Endometrial Epithelial Cells (HEEC)

The endometrial tissue was minced using a scalpel, for 5 minutes, and digested in an enzymatic solution with 0.1 mg/ml DNase I and 0.5 mg/ml collagenase in 10 ml phenol red-free DMEM/F12 for one hour at 37 °C, with intermittent shaking. After one hour, the solution was filtered through a 40 µm cell strainer. Stromal cells passed through the filter and the endometrial gland clumps were retained. The strainer was backwashed with 20 ml DMEM/F12 and centrifuged at 280 g for 5 minutes. The cell pellet was re-suspended in 1 ml 0.25 % trypsin-EDTA and incubated at 37 °C for 10 minutes, to allow dissociation of the glandular clumps. After 10 minutes, 10 ml 10 % DCC medium were added to stop the enzymatic process and the solution was pipetted up and down several times to physically dissociate the gland clumps. Cell suspension was centrifuged at 280 g for 5 minutes, and the pellet re-suspended and seeded in tissue culture dishes. The first media change were undertaken after 60 hours to allow the epithelial cells to attach to the dish. Subsequent media changes were carried out every other day until the cells were confluent.

2.2.1.5 Cell Passage

Cells were washed with phosphate buffered saline (PBS) lifted with 0.25 % Trypsin-EDTA, and incubated for 5 minutes at 37 °C. The flask was gently agitated and cell detachment checked intermittently on the microscope. In order to stop trypsin digestion, 10% DCC medium was added and the cell suspension was centrifuged at 280 g for 5 minutes. Cells were re-suspended, and seeded in tissue culture dishes.

2.2.1.6 Cell Freezing

Once the cells were separated (see 'Isolation of endometrial stromal cells' and 'Isolation of endometrial epithelial cell'), the cell pellets were re-suspended in 900 µl DCC. 900 µl of a 20 % (v/v) DMSO in DCC solution was added dropwise with continuous mixing of the solutions. The solution was mixed by pipetting up and down several times and transferred to a cryovial. Cells were frozen at a rate of 1 °C/minute in a freezing container (Nalgene, Mr. Frosty) with isopropanol, and kept overnight, at - 80 °C before transfer to liquid nitrogen the following day.

2.2.1.7 Cell Thawing

The cryovial was transferred from liquid nitrogen to dry ice. The cryovial was thawed at 37 °C for 4 minutes, and cells were immediately added into a 50 ml Falcon tube with 10 ml pre-heated 10 % DCC media. The solution was centrifuged at 280 g for 5 minutes before the pellet was re-suspended and seeded into a tissue culture dish.

2.2.1.8 Culture of 3T3 Swiss Albino Mouse Fibroblasts

Cells were supplied as frozen cryovial stock. Cells were submitted to the same method described in 'Cell thawing', and were seeded into a T75 flask using DMEM/F12 supplemented with 10 % (v/v) FBS, 1 % (v/v) antibiotic-antimycotic and 2 mM L-glutamine. Media were changed every other day. Cells were passaged when

90 % confluent, using the same method described in 'Cell passage', and were split in one T75 and four 100 mm Petri-dishes. The cells in the Petri-dishes were used for irradiating with UVC light.

2.2.1.9 Irradiation of 3T3 Swiss Albino Mouse Fibroblasts

Cells were irradiated when they reached 60 - 70 % confluency. Media were removed and saved. 10 ml of PBS was added to the Petri-dish and next aspirated. The Petri-dish was irradiated with a 100 J/m² dose of ultraviolet C light (254 nm), using a CX-2000 UVC crosslinker (UVP, Upland, CA, USA). Saved media was added to the cells, and after 3 days, the supernatant was harvested and stored at - 20 °C.

2.2.1.10 Differential Trypsinization for Separation of Feeder Layer and HEEC

Cells were washed with PBS, and incubated in 1 ml 0.25 % trypsin-EDTA for 1 -2 minutes under phase microscopy monitoring. Once the fibroblasts started to detach dishes were gently tapped and the floating feeder cells were removed. Remaining cells were washed in PBS, and 1 ml 0.25 % trypsin-EDTA was added again for the dissociation of HEEC for another 5-7 minutes. Floating HEEC were transferred to 14 ml Falcon tubes and 9 ml 10% DCC medium were added to stop trypsin digestion. Cells were centrifuged at 280 g for 5 minutes, re-suspended in conditioned reprogramming of cell (CRC) medium and seeded in fresh dishes.

2.2.1.11 Decidualization of HESC

Only HESCs between passages 1 and 3 were used for decidualization. The cells were cultured in 10 % DCC media until 90 % confluent. Cells were down-regulated overnight in 2 % DCC phenol red-free DMEM/F12 and the decidualization was carried out the following day. The cells were treated with 0.5 mM 8-bromoadenosine 3',5'-cyclic adenosine monophosphate (cAMP) and 1 µM medroxyprogesterone acetate

(MPA) in 2% DCC phenol-free DMEM/F12. Decidualizing medium was changed every other day.

2.2.1.13 Co-culture of HESC and HEEC

HESC and HEEC were isolated as previously described. Cells were counted with a Luna Automated Cell Counter (Logos Biosystems – South Korea). HESC were seeded into the bottom of 24-well plates at a density of 0.5×10^5 cells/well. HEEC were plated on a 0.4 μ m pore membrane of a transwell cell culture insert at a density of 3×10^5 cells/insert. Different cell types were cultured in separate wells until 90 % confluency. Transwell inserts with the HEEC were placed inside the wells with HESC for co-culture experiments.

2.2.1.14 HEEC Spheroids in Hanging Drops

HEEC were isolated as previously described. Cells were counted. A suspension with 2×10^5 cells/ml in CRC medium supplemented with 2.5 % (v/v) Matrigel was prepared, and 45 μ l/well were seeded in a hanging drop plate. The reservoirs in the hanging drop plates were filled with 1 % agarose gel. 10 μ l media were added to each well every two days. The spheroids were transferred after 4 days, by simple pipetting, into 96-well plates containing 50 μ l ice cold Matrigel/well for treatment. The plates were placed in the incubator at 37 °C for 45 minutes for the Matrigel to set and 100 μ l CRC medium was added into the wells.

2.2.1.15 HEEC Organoids in Matrigel and HGF Supplemented Medium

Isolated cells were counted with a Luna Automated Cell Counter (Logos Biosystems – South Korea), and 0.1×10^5 cells in 50 μ l of ice-cold Matrigel were seeded in 96-well plates. Plates were placed in the incubator at 37 °C for 45 minutes for the Matrigel to set and 100 μ l CRC medium supplemented with 50 ng/ml HGF was added.

2.2.1.16 HEEC Organoids in Matrigel and Expansion Medium

HEEC were isolated as previously described. The final cell pellets were suspended in ice cold Matrigel at a ratio of 1:20 (v:v), and 20 µl drops/well of the Matrigel-cell suspension were plated into 24-well plates. Matrigel was allowed to set at 37 °C for 45 minutes and then 350 µl organoid expansion medium was added. Media were changed every other day.

2.2.1.17 HEEC Organoid Formation Efficiency (OFE) Assay

Frozen HEEC were thawed as previously described. Live cells were counted using Trypan Blue. A suspension with 1,000 live cells per 5 µl ice cold Matrigel was prepared, and 5 µl drops were plated into 96-well plates. The drops were allowed to set at 37 °C for 45 minutes and 100 µl organoid expansion medium supplemented with 10 µM Rock inhibitor Y-27632 was added. Media were changed every 3 days and the number of organoids per well was manually counted after 10 days. At least 3 wells per sample/group were counted and averaged. Organoid forming efficiency was calculated using the formula

$$\text{OFE (\%)} = \frac{\text{number of organoids}}{\text{number of seeded cells}} \times 100.$$

2.2.1.18 Culture of HEEC in Scaffolds

Triacrylate scaffolds cut into 200 µm thick and 10 mm diameter discs were developed in Warwick Chemistry Department by Prof. Neil Cameron and Dr. Ahmed Eissa. The discs were placed in 24-well plates, disinfected with 100 % ethanol, rendered hydrophilic with 70 % ethanol, and washed twice with sterile PBS. The scaffolds were coated to improve cell adherence. To coat the scaffolds, a 0.33 mg/ml fibronectin in PBS solution was used. After the second wash, PBS was aspirated, and 300 µl fibronectin solution was added. The scaffolds were left in the fibronectin for at least 1 hour before cell seeding. HEEC were isolated as previously described and counted.

A suspension of 2.5×10^5 cells in 50 μ l CRC media was carefully added onto the centre of each scaffold disc and incubated for 1 hour at 37 °C. After the incubation, 2 ml CRC medium was added to the wells. Media were changed every other day.

2.2.2 Cell and Tissue Staining

2.2.2.1 Scaffold Haematoxylin-Eosin (H&E) Staining

Media were aspirated from the wells. Discs were washed twice with 2 ml PBS and transferred to a flask with 4 % formaldehyde. Formaldehyde fixed discs were processed and embedded in paraffin and 5 μ m sections were obtained with a microtome. Sections were mounted on a glass slide and left to dry at 60 °C overnight. Slides were re-hydrated by a sequence of 3 baths of xylene for 5 minutes each, followed by 2 baths of 100 % isopropanol for 2 minutes each. Next, slides were dipped in 70 % isopropanol for 2 minutes and rinsed in distilled water for 2 minutes. Slides were incubated with filtered haematoxylin for 1 minute and rinsed with warm running tap water for 15 minutes. They were placed in distilled water for 30 seconds and in 95% ethanol for 30 seconds. Cells were counterstained with eosin-Y for 1 minute. To dehydrate and clear the sections, slides were immersed in a sequence of 2 baths of 95% ethanol, 2 bath of 100% ethanol and 2 baths of xylene (2 minutes each). Distyrene/plasticizer/xylene (DPX) mounting medium was employed for mounting the slides with coverslips.

2.2.2.2 Scaffold Immunostaining

Formaldehyde fixed discs were processed and embedded in paraffin and 5 μ m sections were obtained with a microtome. Sections were mounted onto glass slides and left to dry at 60 °C overnight. Slides were re-hydrated by a sequence of 3 baths of xylene for 5 minutes each, followed by 2 baths of 100 % isopropanol for 2 minutes each. Next, slides were dipped in 70 % isopropanol for 2 minutes, rinsed in distilled

water for 2 minutes, dipped in 10 mM citrate buffer pH 6.0, and placed in an epitope retriever unit for 2 hours. For immunostaining, Novolink Polymer Detection System (Leica) was used. Peroxidase Block is added for 5 minutes, and 0.05 % polysorbate 20 (Tween 20) in tris buffered saline (TBS-T) was used twice for washing. Sections were incubated overnight at 4 °C in primary antibodies diluted in 0.05 % TBS-T. The slides were again rinsed twice with TBS-T and incubated with Post Primary Block for 30 minutes. Two more 0.05 % TBS-T washes were carried out and the slides were incubated for 30 minutes with Novolink Polymer Solution. Slides were rinsed with 0.05 % TBS-T, incubated for 5 minutes with Diaminobenzidine Chromogen and rinsed with water for 5 minutes. Slides were counterstained with haematoxylin.

2.2.2.3 Immunofluorescence

Cell monolayers were grown in glass-bottom Petri-dishes until 90 % confluent. Organoids in Matrigel were cultured for 7 days. Culture media were aspirated, cells were washed in PBS and fixed in 4 % formaldehyde. Formaldehyde was aspirated and washed clear with PBS 5 minutes. Cells were permeabilized with 0.1 % Triton X-100 for 1 hour at room temperature, washed with PBS for 5 minutes and incubated in 1% BSA / PBS (v/v) for 1 hour to block nonspecific binding of antibodies. Cells were incubated overnight with primary antibodies diluted 1/100 in 1% (w/v) BSA/PBS at 4 °C, washed with 1% BSA/PBS and incubated with secondary antibodies diluted 1/200 in 1 % BSA/PBS at 4 °C for 2 hours in the dark. A new 1% BSA / PBS wash was carried out, and cells were coverslipped with mounting medium containing DAPI for nuclear counterstaining. The cells and organoids were imaged with a confocal microscope.

2.2.3 xCELLigence Real-Time Cell Analysis (RTCA)

An xCELLigence RTCA DP system (ACEA Biosciences, San Diego, CA, USA) was used for proliferation assays. This system employs microelectrodes integrated into

the bottom of microwell plates (E-plates) to measure electric impedance. A current between the negative and positive electrodes pass through an electrically conductive solution (culture medium). Adherent cells at the electrode-solution interface hinder the electron flow. The impedance increases proportionally to the number of attached cells and is displayed as cell index values. This index provides an estimative of cell proliferation and attachment. The instrument is placed inside a humidified incubator at 37 °C with 5% CO₂.

The instrument was set-up for data acquisition parameters: impedance recording intervals and duration of the experiment. Cells were isolated as previously described. Wells were loaded with 10,000 cells in 200 µl of the indicated culture media. E-plates were placed in the RTCA system and allowed to settle for 15 minutes before running the experiment. Treatment/cell type groups were loaded in duplicates. Cell index values were plotted against time (hours) for producing the graphs.

2.2.4 Glycogen Measurement in Endometrial Gland Organoids

A colorimetric assay (Abcam) was used for measuring the glycogen levels in endometrial gland organoids in 24-well plates. In this assay, glycogen is hydrolysed into glucose, which is oxidised. Oxidised glucose renders a colourless probe to a coloured compound with strong absorbance at 450 nm.

Media from organoids in Matrigel were removed. Organoids were washed twice with 500 µl/well of PBS and 400 µl/well of Cell Recovery Solution (Corning) was added. Plates were placed in the fridge for 1 hour to melt the Matrigel. Organoids in Cell Recovery Solution were transferred to 14 ml Falcon tubes and 10 ml of ice-cold PBS was added. Tubes were centrifuged at 280 g for 5 minutes and another PBS wash was performed to remove residual Matrigel. PBS was removed and 50 µl/well of Glycogen Hydrolysis Buffer was added. Cells were homogenised for 10 minutes on ice. Lysates were transferred to Eppendorf tubes and centrifuged at 16,000 g for 10

minutes. Supernatants were collected and 5 µl added to 45 µl of Glycogen Hydrolysis Buffer in a 96-well clear flat bottom plate (1/10 dilution samples). Glycogen standards containing 0, 0.4, 0.8, 1.2, 1.6, and 2 µg/50 µl were prepared in the same plate. Standards and samples were loaded in duplicate. Background control samples were set using pooled samples. 2 µl Hydrolysis Enzyme Mix were added to standards and samples and the plate was incubated at room temperature for 30 minutes. A master mix containing 44 µl of Glycogen Development Buffer, 2 µl of Development Enzyme Mix and 2 µl of Probe per sample was prepared, and 48 µl were added to standards and samples. A master mix containing 46 µl of Glycogen Development Buffer, 2 µl of Development Enzyme Mix and 2 µl of Probe per sample was prepared, and 50 µl was add to background control wells. The plate was incubated at room temperature for 30 minutes and was read at 450 nm with a microplate reader. A standard curve was generated and used to calculate the amount of glycogen in the samples. The amount of glycogen in µg was normalized to the total protein content in the samples (measured by Bradford assay).

2.2.5 mRNA Analysis

2.2.5.1 Primer Design

Primers were initially designed using online tools such as Roche Universal Probe Library and Primer-Blast or Primer3. Corrections were made to fit the primers within the following parameters, wherever possible:

- a) Spanning intron amplicon
- b) Amplicon length between 65 and 125 base pairs.
- c) Melting temperature between forward and reverse primer ≤ 1 degree.
- d) At least two C or G amongst the last five bases at the 3' end.
- e) No more than 4 of the same base in a run.
- f) Primer length between 18 - 24 base pairs

Primer specificity was tested using the online tool Basic Local Alignment Search Tool (BLAST). Secondary structure were checked using the online tool OligoAnalyzer 3.1.

Links:

Roche Universal Probe:

<https://lifescience.roche.com/shop/CategoryDisplay?catalogId=10001&tab=Assay+Design+Center&identifier=Universal+Probe+Library&langId=-1>

Primer-Blast:

http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome

Primer3: http://biotools.umassmed.edu/bioapps/primer3_www.cgi

Basic Local Alignment Search Tool (BLAST):

http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome

OligoAnalyzer 3.1:

<https://www.idtdna.com/calc/analyzer>

2.2.5.2 Primer Optimization

Primers pairs were first tested by melt curve analysis. Pairs were used to amplify random cDNA by RT-qPCR at a 300 nM concentration. Melting curves were analysed for primer dimer and amplicon melting temperature. Amplified cDNA was saved and used for agarose gel analysis. Gels (3 %) were prepared dissolving 3 g agarose powder in 100 ml boiling TBE and 2 µl ethidium bromide. Gels were transferred to casting plates inserted with combs while still liquid, and allowed to set. Combs were removed, and plates were placed into the electrophoresis tank with TBE. 20 µl cDNA was mixed with 4 µl DNA loading dye (6x) and 10 µl cDNA-dye solution and 3 µl Hyper Ladder V (Bioline) were applied into the wells. Gels were run at 100 V until halfway

line. Purified products were visualized in a G:Box (Syngene, Cambridge, UK) and amplicon sizes were confirmed.

Table 2.2 Primers utilized in experiments

Gene	Forward primer	Reverse primer
<i>ALKBH7</i>	agacgctgagccgagaact	gacttctctgtctctcggaag
<i>ESR1</i>	ggctacatcatctcggtcc	cttcagggtgctggacagaa
<i>F2RL1</i>	atcctgctagcagcctctc	gtgggatgtgcatcaacctt
<i>FAM89A</i>	cgctgctccgcaaagagat	aggctgtacagttggcagag
<i>LHCGR</i>	tgccttgacaacctcctcaat	gctccgggctcaatgtatct
<i>MFAP4</i>	ggcgtgtacctcatctacc	gagccattgaatctcttctgga
<i>NID2</i>	tggctggctcttgccttagaa	atgggtaaaggcagcacctg
<i>PAEP</i>	gagcatgatgtgccagtacc	tgatgaatccctgcatgatctc
<i>PGR</i>	ctggcatggctccttgag	ttcatttggaacgccactg
<i>PRL</i>	aagctgtagagattgaggagcaaac	tcaggatgaacctggctgacta
<i>PTGS2</i>	ccagcacttcacgcatcagt	gggtggacttaaatcatatttacggt
<i>SCNN1A</i>	catccctggaggaggaca	tggtggaagtgagagtaattcg
<i>SCNN1B</i>	caccaatatcaccctgagcag	gagagcagccagacgatgt
<i>SCNN1G</i>	gagtgcgtgccaatcagg	tgtctggaagcatgaatgaagg
<i>TERT</i>	cggtgtgcaccaacatcta	gcacacatgcgtgaaacct
<i>TIMP3</i>	gctggagggtcaacaagtacca	cacagccccgtgtacatct
<i>ZNF319</i>	acctcatgctgccacaaagtg	aaggctctgcctacaggaca
<i>L19</i>	gcggaagggtacagccaat	gcagccggcgcaaaa

2.2.5.3 DNA Gel Extraction of PCR Amplified Products

PCR products from tested primers were extracted from 1 % agarose gel to generate standard curves. Gels were prepared dissolving 1 g agarose powder in 100 ml boiling TBE and 2 µl ethidium bromide. Gels were transferred to casting plates inserted with combs while still liquid, and allowed to set. Combs were removed, and plates were

placed into electrophoresis tanks with TBE. 80 µl cDNA was mixed with 16 µl DNA loading dye (6 x). 3 µl Hyper Ladder V (Bioline) were applied into one of the wells and cDNA-dye solution was split in the remaining wells. Gels were run at 100 V till half line. Purified products were visualized in a UV cabinet and excised with scalpels. For DNA extraction, a gel extraction kit (Qiagen) was used. Gel slices were weighed, after the addition of 3 volumes of Buffer QG, they were incubated at 50 °C for 10 minutes to dissolve gels. 1 gel volume of isopropanol was added, solutions were mixed, and applied to Qiagen columns. Columns were centrifuge at 17,000 g for 1 minute. Flow-through were discarded, and 500 µl Buffer QG were applied to the columns. Columns were centrifuged at 17,000 g for 1 minute and 750 µl Buffer PE added. After a further 17,000 g centrifugation for 1 minute the columns were transferred to fresh 1.5 ml microcentrifuge tubes. To elute DNA, 30 µl Elution Buffer was applied to the centre of column membranes, incubated at room temperature for 1 minutes and centrifuged at 17,000 g for 1 minute. Final DNA concentrations were measured using a spectrophotometer.

2.2.5.4 Primers Standard Curves for Calculation of Efficiency

Gel extracted cDNA was serially diluted from 100 pg/µl to 10 ag/µl using serial 1/10 dilutions, before amplification in triplicate by RT-qPCR with test primers. To generate the standard curve, the log of the concentrations of cDNA were plotted against the average Ct value from triplicates. The following formula was used to calculate primers efficiency:

$$\text{Primer efficiency} = 10^{\frac{-1}{\text{gradient of the line}}}$$

2.2.5.6 RNA Isolation from Cells in Culture

Cells in culture were washed with PBS, and harvested by scraping, after the addition of phenol-guanidinium thiocyanate monophasic solution (Stat-60). Homogenates

were transferred to RNase-free Eppendorf tubes, and 20 % volume of ice cold chloroform added before vigorously vortex for 15 seconds. Tubes were centrifuge at 16,000 g at 4 °C for 30 minutes to separate the upper aqueous phase, where the RNA remains, from the organic phase at the bottom, where DNA and protein stay. The aqueous phase was transferred to RNase-free Eppendorf tubes containing half of the original STAT-60 volume of ice-cold isopropanol and 20 µg glycogen. Tubes were thoroughly vortexed and stored at - 80 °C for at least 30 minutes (maximum 24 hours) to precipitate the RNA. Tubes were thawed on ice and centrifuged at 16,000 g at 4 °C for 15 minutes. RNA pellets were washed twice with 500 µl of 75 % (v/v) ethanol in RNase free-water, and allowed to dry for 2 minutes, before resuspension in TE buffer pH 8.0. RNA concentration and purity were measured using a spectrophotometer. RNA purity was considered satisfactory when the absorbance ratio at 260/280 \geq 1.8.

2.2.5.7 RNA Isolation from Cells in Matrigel

This method was used for spheroids and organoids in 3D culture. Culture media were removed and wells were washed twice with PBS. Ice cold Cell Recovery Solution (Corning) was added to wells and plates were stored at 4 °C for one hour to melt the Matrigel. Suspensions containing the cells were transferred to 14 ml Falcon tubes containing ice-cold PBS. Wells were examined at the microscope for remaining spheroids or organoids and if necessary were scraped. Tubes were centrifuged at 280 g at 4 °C for 5 minutes. Two more washes with ice-cold PBS were carried out. 400 µl Stat-60 were added to the pellets and tubes were placed on the shaker at room temperature for 10 minutes. Homogenates were transferred to RNase-free Eppendorf tubes, 20 % volume of ice cold chloroform was added, and tubes were vigorously shaken for 15 seconds. Tubes were centrifuge at 16,000 g at 4 °C for 30 minutes to separate the upper aqueous phase, where the RNA remains, from the organic phase at the bottom, where DNA and protein stay. The aqueous phase was transfer to

RNAse-free Eppendorf tubes containing half of the original STAT-60 volume of ice-cold isopropanol and 20 µg glycogen. Tubes were thoroughly vortexed and stored at - 80 °C for 30 minutes to precipitate the RNA. Tubes were thawed on ice and centrifuged at 16,000 g at 4 °C for 15 minutes. RNA pellets were washed twice with 500 µl 75 % (v/v) ethanol in RNAse free-water. Pellets were allowed to dry for 2 minutes and were re-suspended in TE buffer pH 8.0. RNA concentration and purity were measured using a spectrophotometer.

2.2.5.8 RNA Isolation from Organoids in Organoid Forming Efficiency Assay

RNA was isolated using a kit (Ambion). Media from organoids were aspirated and 100 µl Lysis Buffer were added. Plates were shaken at room temperature for 10 minutes, and lysates were collected into RNAse-free Eppendorf tubes. Half volume of 100% ethanol was added to lysates, mixtures were briefly vortexed, loaded onto Micro Filter Cartridge Assemblies, and centrifuged for 15 seconds at 16,000 g. Collection Tubes were emptied, 180 µl Wash Solution 1 were added, and Micro Filter Cartridge Assemblies were centrifuged for 15 seconds at 16,000 g. 180 µl Wash Solution 2/3 were added and Micro Filter Cartridge Assemblies were centrifuged for 15 seconds at 16,000 g. A second 180 µl aliquot of Wash Solution 2/3 was added and centrifuged for 15 seconds at 16,000 g. Collection Tubes were emptied, and the assemblies were centrifuged for 1 minute at 16,000 g to remove the residual fluid. Micro Filter Cartridges were transferred to Elution Tubes, 10 µl Elution Solution at 75 °C were applied to the centre of filters, and assemblies were stored at room temperature for 1 minute. Assemblies were centrifuged for 30 seconds at 16,000 g to elute RNA from filters. A second 10 µl aliquot of Elution solution was added, and after one minute at room temperature assemblies were centrifuged again for 30 seconds

at 16,000 g. Eluates were transferred to RNase-free Eppendorf tubes and frozen at -80 °C.

2.2.5.9 Complementary DNA (cDNA) Synthesis from mRNA

A reverse transcription kit (Qiagen) was used for cDNA synthesis. 1 µg of template RNA, 2 µl of 7 x gDNA Wipeout Buffer and nuclease-free water to complete 14 µl were mixed in pre-chilled RNase-free Eppendorf tubes. Tubes were vortexed, centrifuged briefly and incubated at 42 °C for 2 minutes to remove the genomic DNA. 4 µl RT Buffer (5 x), 1 µl RT Primer Mix and 1 µl Quantiscript Reverse Transcriptase were added to each tube, vortexed, and briefly centrifuged. Control sample without the reverse transcriptase were prepared, using 1 µl of water instead of the enzyme. Tubes were incubated at 42 °C for 30 minutes. To inactivate the reverse transcriptase tubes were incubated at 95 °C for 3 minutes. Final cDNA was diluted 1/5 by adding 80 µl nuclease free-water. Samples were assayed immediately or stored at – 20 °C.

2.2.5.10 Real-time Quantitative Polymerase Chain Reaction (RT-qPCR)

A real-time PCR instrument (7500 Real-Time PCR System, Applied Biosystems, CA, US) was used for amplification and quantification of genes of interest. Primers at 300 nM concentration, 10 ng cDNA template and a ready-made master mix (SYBR green I) containing deoxynucleotides, magnesium, DNA polymerase and a fluorescent dye was added at a volume of 20 µl / well in 96-well optical PCR plates. Plates were centrifuged at 1,100 g for 3 minutes. cDNA samples were tested in triplicates. Non-template controls were used, replacing the cDNA by 1 µl nuclease free-water. A housekeeping (*RPL19*) gene was always used to normalize between the different samples. For amplification the instrument was set-up for the following thermocycling sequence:

- a) 95 °C for 10 minutes (pre-cycling stage)
- b) 95 °C for 15 seconds
- c) 60 °C for 1 minute

A fluorescent signal is released when the SYBR green is incorporated into the DNA double strand and it is measured by the instrument in each cycle. As the amplified DNA accumulates the fluorescence increases. Therefore an amplification curve is generated by the instrument. A threshold is assigned within the exponential phase to define a cycle threshold (Ct) value. A Ct value is the number of cycles required for the curve to cross the given threshold.

For the dissociation curve the sequence was:

- a) 95 °C for 15 seconds
- b) 60 °C for 1 minute
- c) 95 °C for 30 seconds
- d) 60 °C for 15 seconds

The Pfaffl method was chosen for the analysis (Pfaffl, 2001). This method compares the difference between Ct values with PCR reaction efficiency correction. The formula used for this method is: Expression value = $E^{-\Delta Ct}$

2.2.6 Protein Analysis

2.2.6.1 Protein Extraction and Quantification (Bradford assay)

A 1x radioimmunoprecipitation assay (RIPA) lysis buffer solution with protease inhibitor was prepared and stored in ice. Culture medium was aspirated and cells were washed with ice-cold PBS, before addition of RIPA buffer. Cells were scraped and lysates were transferred to sterile microcentrifuge tubes. Tubes were centrifuged at 16,000 g for 10 minutes at 4 °C to eliminate cell debris. Supernatants were transferred to sterile microcentrifuge tubes and stored at - 80 °C. For nuclear proteins,

the RIPA buffer was replaced by whole cell extraction buffer also with protease inhibitor.

Protein concentration was determined by the Bradford method (Bradford, 1976). A 10 mg/ml bovine serum albumin (BSA) stock solution was prepared and was used for protein standard dilutions. The BSA dilutions were 0, 0.5, 1, 2, 4, 6, 8 and 10 µg/ml. Tested protein samples were diluted 1/400 (v/v) in distilled water. A protein dye reagent concentrate containing Coomassie Brilliant Blue dye was added to the standard dilutions and to tested samples at a ratio of 1/5. This dye binds the proteins, forming a complex that absorb light at 594 nm. Tubes were incubated at room temperature for 15 minutes, vortexed and loaded in duplicates into a 96-well assay plate. Plates were read at 595 nm. Standard curves were generated from the optical density of the BSA standards and were used to calculate the protein concentration of tested samples.

2.2.6.2 Polyacrylamide Gel Electrophoresis

The percentage of acrylamide in the resolving gels depended on the protein size. For proteins ranging from 14 kDa to 200 kDa, 12 % gel were chosen. Stacking gels contained 4 % acrylamide were prepared to pH 6.8. Resolving gels were prepared to pH 8.8. Disposable cassettes were used for gel preparation. Tetramethylethylenediamine (TEMED) and 10 % ammonium persulfate (APS) were applied to induce polymerisation. Resolving gels were added into the cassettes and coated with isopropanol. Once gels were set, the isopropanol was removed, stacking gels were added, and combs inserted.

A mixture containing 20 µg total protein, 10 % dithiothreitol and 25 % 4 x lithium dodecyl sulphate (LDS) buffer was prepared and heated to 95 °C for 10 minutes to denature the protein of interest, giving the antibody access to its epitope.

Gels were placed into electrophoresis tanks and covered with running buffer. Protein mixtures and a pre-stained protein ladder were loaded into the wells. The current was set to 230 mA and run at 125 V until the ladder reached the bottom of the gels. Separated proteins were subjected to membrane transfer.

2.2.6.3 Western-Blotting

Proteins run in gels were transferred to a polyvinylidene fluoride (PVDF) membrane. Membranes were activated with a 30 second methanol rinse. Gels and membranes were sandwiched between filter papers and blotting pads on each side and stacks were placed between plate electrodes in electrophoresis tanks. Tanks were filled with transfer buffer, and were run at 230 mA and 125 V for 2 hours.

After the transfer, membranes were dried, reactivated with methanol, rinsed with tris-buffered saline (TBS) and blocked in 5 % milk in TBS for one hour. Next, membranes were incubated with primary antibodies diluted in milk at 4 °C with constant movement. Incubations with β -actin antibody lasted 2 hours. Incubations with all the other antibodies were carried out overnight (Table 2.1)

Membranes were rinsed with 0.2% TBS-T, followed by one 15 minute and three 5 minute washes in 0.2% TBS-T. Membranes were then incubated with secondary antibodies for 1 hour at room temperature. Secondary antibodies conjugated to horseradish peroxidase (HRP) and matching the species of primary antibodies were chosen. Another round of TBS-T washes (15 minutes, 5 minutes and 5 minutes) was performed and TBS was used for the final wash. Chemiluminescent detection of antigens conjugated to HRP labelled antibodies was accomplished with an enhanced chemiluminescence (ECL) detection system and using a G:Box (Syngene, Cambridge, UK).

Table 2.3 Primary antibodies and dilutions utilized in experiments

Primary antibodies	Dilutions
Cytokeratin 18	1/1,000
Vimentin	1/1,000
p53	1/3,000
p16	1/1,500
Lamin B1	1/1,000
HMGB2	1/500
Hist3meK9	1/1,000
B-actin	1/20,000

2.2.7 Senescence Measurement

2.2.7.1 Senescence-Associated β -Galactosidase (SA β gal) staining

A commercially available kit (Cell Signaling) was used for SA- β -gal staining. Cells were cultured until 90 % confluent. Media were removed and cells were washed with PBS. Fixative solution was added into the dishes and were incubated at room temperature for 10 minutes. Dishes were rinsed twice with PBS. An X-gal stock solution (20 x) was prepared dissolving 20 mg of X-gal in 1 ml dimethylformamide. β -gal staining solution was prepared according to the following recipe:

Distilled water: 837 μ L

10 x staining solution: 93 μ L

Staining supplement A: 10 μ L

Staining supplement B: 10 μ L

X-gal stock solution (20 x): 50 μ L

Dishes were incubated overnight with β -gal solution at 37 °C in a dry incubator (no CO₂). Cells were imaged in the following day or were stored in 70 % glycerol at 4 °C.

2.2.7.2 Senescence Associated β -gal Activity Measurement

A commercially available fluorometric format kit (Cell Biolabs) was used for SA β -gal activity measurements. Lysis Buffer was prepared immediately before use and proteinase inhibitor added. Assay Buffer (2 x) was prepared by adding 0.704 μ l β -mercaptoethanol in 1000 μ l 2 x Reaction Buffer, and then adding 50 μ l 20 x SA- β -gal Substrate in 950 μ l of this solution (2 x Reaction Buffer containing β -mercaptoethanol).

Cells were seeded in 24-well plates. Culture media were aspirated and cells were washed with cold PBS. After aspirating the PBS cells were incubated with Lysis Buffer at 4 °C for 5 minutes. Lysates were transferred to microcentrifuge tubes and centrifuged at 4 °C for 10 minutes to remove debris. Supernatants were collected and stored at - 80 °C. Total protein concentration of lysates were determined by Bradford assay. 50 μ l of cell lysates were transferred to 96-well plates, 50 μ l 2 x Assay Buffer were added to the wells, and plates were incubated at 37 °C for 1 hour in the dark, without CO₂. Negative controls were set up replacing cell lysates for 50 μ l Lysis Buffer. 50 μ l of the reaction mixture were removed from the wells and transferred in duplicate to black 96-well plates in duplicate. To stop reactions, 200 μ l Stop Solution was added into the wells. Fluorescence was read at 360 nm excitation and 465 nm emission. Fluorescence intensity units (FU) of the duplicates were averaged, the value was divided by the negative control, and were normalized dividing the result by sample total protein amount (mg).

2.2.8 Enzyme-Linked Immunosorbent Assay (ELISA)

2.2.8.1 Prostaglandin E₂ (PGE₂)

A competitive immunoassay kit for quantitative determination of PGE₂ (Thermo-Fisher) in cell culture supernatant was utilized. PGE₂ amount in standard dilutions or samples compete with an alkaline phosphatase conjugated PGE₂ (PGE₂-AP) for the specific primary antibody. The plate is supplied covered with the secondary antibody. Unbound antigen is removed by washing the plate. A chromogenic substrate is added and PGE₂-AP cleaves this substrate triggering a chromogenic signal. As the competitive binding is concentration dependent the signal output is inversely correlated with the amount of antigen in the sample.

Cell culture supernatant was retrieved and centrifuged to remove debris and stored at - 80 °C until use. Eight standard dilutions were prepared using 2% DCC medium: 5,000; 2,500; 1,250; 625; 313; 156; 78.1 and 39.1 pg/ml. Cell culture supernatant samples were diluted 1/2. Standards and supernatant samples (100 µl/well each) were loaded into the wells in duplicate. PGE₂-AP and PGE₂ antibody (50 µl/well each) were added and plates were incubated at room temperature on a plate shaker for 2 hours at 500 rpm. Plates were washed 3 times with 400 µl Washing Buffer and 200 µl Substrate Solution. Plates were incubated at room temperature for 45 minutes and 50 µl Stop Solution were applied to stop the reaction. A plate reader was used at 405 nm with correction at 541 nm for determining the optical density. A standard curve based on the standard dilutions optical density was generated. The concentration of PGE₂ in the samples were calculated using the standard curve. The concentration of PGE₂ in pg/ml was normalized to the total protein content in the samples (measured by Bradford assay).

2.2.9 Laser Microdissection (LMD)

2.2.9.1 LMD of Endometrial Glands

Snap-frozen human endometrial biopsies from secretory phase were used for LMD. The frozen samples were embedded in optimal cutting temperature compound (OCT), and 10 µm sections were obtained with a cryostat. The sections were mounted on polyethylene naphthalate membrane slides and stained with cresyl-violet and eosin-Y. The slides were immersed in 95 % (v/v) ethanol in nuclease-free water for 30 seconds. A mixture of 70 % cresyl-violet and 30 % eosin-Y (v/v) was used to stain for 40 seconds. The slides were sequentially immersed in 95 % ethanol for 30 seconds, 100 % ethanol for 30 seconds and xylene for 5 minutes. A vacuum desiccator was employed to dry the slides.

Capture of endometrial glands was carried out for no more than 30 minutes, to avoid excessive RNA degradation. Membrane slides were overlaid by a glass slide so that the tissue section was interposed between the glass and the membrane. Endometrial glands were dissected with a laser microdissection unit coupled to an inverted microscope and a 3-CCD camera. Glands were retrieved by isolation diffuser caps.

2.2.9.2 RNA Isolation, DNase I Digestion and Assessment of Laser Capture Samples

An RNA isolation kit for LMD (Ambion) and an on-column DNase I digestion (Omega Bio-tek) kits were used. The captured samples were stored in the provided Lysis Solution at - 80 °C, until the RNA isolation. DNase I digestion solution was prepared by mixing 1.5 µl DNase I (20 Kunitz/µl) with 73.5 µl Digestion Buffer for each sample. Caps were thawed on ice and incubated at 42 °C for 30 minutes. After vortexing and briefly centrifuging to collect the fluid at the bottom of the cap, 3 µl LCM Additive and 129 µl 100 % ethanol were added. Lysate/ethanol mixtures were loaded onto

prepared Micro Filter Cartridge Assemblies (columns) and centrifuged at 10,000 g for 1 minute. The DNase I digestion solution (75 μ l/sample) was added into the columns which were incubated at room temperature for 15 minutes. 180 μ l Wash Solution 1 were added and columns were centrifuged at 10,000 g for 1 minute. 180 μ l Wash Solution 2/3 were added and columns were centrifuged at 16,000g for 30 seconds. A further wash with 180 μ l of Wash Solution 2/3 was performed and flow through was discarded. Columns were replaced into the same collection tubes and centrifuged at 16,000 g for 1 minute to remove residual fluid. Columns were transferred to Micro Elution Tubes, 10 μ l Elution Solution at 95 °C were applied to the centre of the filters. A 5 minute incubation at room temperature was followed by a centrifugation at 16,000 g for 1 minute. Another 10 μ l Elution Solution at 95 °C was applied to the centre of the filters. After a 5 minute incubation at room temperature, another centrifugation at 16,000 for 1 minute was performed. The eluted RNA was transferred to a nuclease-free tube and stored at - 80 °C until the RNA assessment.

RNA integrity and concentration analysis were performed by the Warwick Genomics Facility at Warwick Life Sciences School using a 2100 Bioanalyzer instrument (Agilent, CA, USA) and an Agilent RNA 6000 Pico chip. RNA integrity number (RIN) and the concentration were determined by the Bioanalyzer 2100 Expert software (Agilent, CA, USA). RNA samples with a RIN \geq 6 and RNA ratio [28s/18s] \geq 1.0 were deemed suitable for sequencing.

2.2.10 RNA Sequencing

2.2.10.1 Library Preparation

Library preparations were performed by Warwick Genomics Facility. Ovation RNA-Seq System V2[®] (Nugen Technologies) was used for cDNA synthesis and amplification. First strand cDNA was prepared using a DNA/RNA chimeric primer mix and reverse transcriptase. This resulted in cDNA/mRNA hybrid molecules containing

a RNA sequence at the 5' end of the cDNA strand. Priming sites for DNA polymerase were created by fragmenting the mRNA within the cDNA/mRNA complex, in order to synthesise a second DNA strand. Next the cDNA was amplified using DNA/RNA chimeric SPIA primers and DNA polymerase. RNA in the 5' end of the cDNA strand was removed from the hybrid molecule by RNase H.

Libraries were produced using Ovation Ultralow System V2 1-16 (Nugen Technologies) in four steps: fragmentation of cDNA double strand, end repair to generate blunt ends, ligation of the adapters with barcoding of libraries and amplification by PCR.

2.2.10.2 RNA Sequencing

The libraries were sent to Wellcome Trust Centre for Human Genomics – High Throughput Genomics (Oxford, UK) for double end next generation sequencing using Illumina HiSeq and with a reading length of 100 base pairs, producing 150 million pair reads.

2.2.10.3 RNA Sequence Data Analysis

Data analysis was carried out in collaboration with Dr. Pavle Vrljicak. Transcriptomic maps of single-end reads were generated using Bowtie-2.2.3, Samtools-0.1.19, and Tophat-2.0.12 against the University of California Santa Cruz (UCSC) hg19 reference transcriptome (2014) from the Illumina Genomes resource using the fr-firststrand setting. Transcript counts were assessed by HTSeq-0.6.1 using the reverse strand setting and intersection non-empty mode and counts were assigned to gene IDs. Transcripts per million were calculated using the method described by Wagner *et al.* (2012). Count data from the TopHat-HTSeq pipeline were analyzed using DESeq2.

2.2.11 Data Mining

Databases from GEO Datasets were mined for expression of several genes for comparisons with our RNA sequencing findings. The utilized databases were:

GSE4888 – Molecular phenotyping of human endometrium

GSE6364 – Gene profiling of endometrium reveals progesterone resistance and candidate genetic loci in women with endometriosis

2.2.12 Statistical Analysis

GraphPad Prism 6 software (GraphPad Software, CA, USA) was used for statistical analysis. Two-tailed paired or two-sample *t*-tests were applied for comparisons between two groups. One-way analysis of variance (ANOVA) with Holm-Sidak test correction was applied for comparisons between more than two groups. Gaussian distribution was verified with D'Agostinho-Pearson and Shapiro-Wilk test. Kruskal-Wallis test (non-paired groups) and Friedman test (paired groups) were chosen for more than two groups with non-parametric distributions. Mann-Whitney *U* test was used for comparing two unpaired groups and Wilcoxon signed-rank test for paired groups. P-values < 0.05 were considered statistically significant.

Chapter 3

Isolation, Culturing and Reprogramming of Primary Human Endometrial Epithelial Cells, and Their Responses to Differentiation Signals

3.1 Introduction

The culture of HEECs allows for studying the function of this compartment in the implantation process, as well as the effect of the endocrine and paracrine cues, coming from the underlying stromal cells, the ovary and the implanting blastocyst. However, since the first attempts to culture isolated HEECs, researchers have struggled to maintain long term functional cells that mimic the *in vivo* responses to differentiation and proliferation signals (Liszczyk et al., 1977, Kirk et al., 1978, Bongso et al., 1988). Once in culture, HEECs rapidly undergo proliferation arrest and become senescent (Kyo et al., 2003, Valentijn et al., 2015). Furthermore, like most epithelial cells, they lose their specialized properties when cultured as monolayers, due to a process known as dedifferentiation, and become unresponsive to ovarian steroid hormones (Kleinman et al., 1983, Classen-Linke et al., 1997).

During the secretory phase of the menstrual cycle, increasing levels of P4 inhibit proliferation of HEECs, counteracting the mitogenic effects of E2 (Pierro et al., 2001). In addition to being anti-mitogenic, P4 is also responsible for inhibiting telomerase activity in the endometrium. Tanaka et al. (1998) demonstrated that the lowest endometrial telomerase activity occurs in the late secretory phase, when compared to proliferative phase. Using *in situ* RNA hybridization, these authors also identified the endometrial glands as the main source of telomerase. The highest expression was detected in GE during the proliferative phase. This may explain the lack of proliferation of HEECS derived from secretory phase when compared to proliferative phase (Fleming, 1999). Taken together, these data reflect the hurdles of culturing HEECS isolated from biopsies taken during the secretory phase of the menstrual cycle.

An alternative approach is to adopt cell lines derived from endometrial cancers to study HEECs. There are relatively cheap commercially available options. These cells,

such as Ishikawa cells, HEC-1A, HEC-1B, HES, RL-92, ECC-1 are easy to culture, fast growing, provide an endless source of cells, and are ease to replace in case of contamination. However, this approach comes with some risks. Frequent genotypic and phenotypic drift during continuous culture has been described (Burdall et al., 2003). Cell lines are susceptible to contamination by several pathogens, especially mycoplasma (Burdall et al., 2003). Selection of subpopulations of cells within the cultures have been described. Several concerns have been manifested in recent years, including the contamination, misidentification and redundancy in cell lines (Burdall et al., 2003). It has been reported that wrong cell lines have been used by researchers (Geraghty et al. 2014). This problem also affects endometrial cell lines (Korch et al., 2012). Nichida (2002) has already raised this issue regarding Ishikawa cells. But the main disadvantage of these models is the lack of knowledge on the differences between cell lines and primary cells. Therefore I opted not to use endometrial epithelial cell lines.

The inability to passage and expand adult epithelial cells that retain lineage commitment, and keep normal growth and differentiation capacity, led to the development of methods for cell immortalization. The use of oncogenes such as SV40 large T antigen (Merviel et al., 1994) or E6/E7 proteins from oncogenic human papillomavirus (Kyo et al., 2003), although successfully avoiding premature senescence of the cultured cells, results in defects in p53 and Rb regulatory pathways. Somatic cells have been reprogrammed into induced pluripotent stem cells (iPSC), prolonging their life span (Saha et al., 2009). Unfortunately the method has been shown inefficient, and is dependent on the transfection of exogenous gene, which could induce changes in genome and antigenicity (Lister et al., 2011, Taylor et al., 2011). A technique using a feeder-layer of irradiated fibroblasts and a Rho-associated kinase inhibitor (ROCK) inhibitor rapidly achieved immortalization of human keratinocytes, which maintain their original karyotypes (Liu et al., 2012). The

use of feeder layers of lethally irradiated mouse fibroblasts for epithelial cell culture was developed by Rheinwald and Green (1975) and has been widely used for serial culturing many types of epithelial cells. The cells in the feeder layer release growth factors, cytokines and extracellular proteins into the culture medium, promote attachment, and remove toxic or inhibitory factors from the culture medium (Lhames et al., 2015). Nevertheless, the method engenders some problems. Synchronization of the feeder layer with the seeding of the target cells can be laborious. Ionizing radiation is not widely available, brings professional risks, and demands a dedicated tissue culture facility. There is also the risk of infection of the target cells with mouse retrovirus, or the transmission of damaged genetic material (Amit et al., 2003). The separation of the feeder layer from the target cells can be cumbersome. A more recent study has indicated that media conditioned by irradiated mouse fibroblasts has the same effect on epithelial cells as co-culture with irradiated feeder layers (Palechor-Ceron et al., 2013).

This chapter explores culturing of primary HEECs and its limitations. Here I report on a feeder-free method for conditionally reprogramming HEECs and investigate the responses of these cells to some signals from the ovaries, the endometrial stroma and the implanting blastocyst.

3.2 Results

3.2.1 Isolation of HEECs and HESCs from Mid-Secretory Endometrium

To isolate purified HEECs and HESCs, I used a method that involved mincing the endometrial tissue, followed by digestion in collagenase and DNase solution, and filtering of the digested tissue through a mesh sieve, and backwash of the sieve. Stromal cells in the flow-through were pelleted, re-suspended, and seeded. Retained glandular clumps were retrieved by backwashing the sieve, and were seeded into the dishes. Unfortunately, this approach often resulted in floating glandular clumps and very few attached cells. To solve this problem, I added a further dissociation step with trypsin-EDTA for 10 minutes to dissociate the glandular clumps. Using this method, it was possible to produce single cell solutions and HEEC cultures that reached confluence, when seeded in a high density (Figure 3A). To test the purity of the cultures, I analysed the protein expression of an epithelial marker (cytokeratin 18; CK18) and a mesenchymal marker (vimentin) in the cell lysates, using Western-blot. The expression of CK18 was marked in HEEC cultures and undetectable in HESC cultures. Conversely, vimentin was highly expressed in HESC cultures and was not observed in HEEC cultures (Figure 3B). In order to further assess purity, the cells were fixed and stained with CK18 and vimentin. Confluent Ishikawa cells were also fixed and stained for CK18 to serve as a positive control for HEECs. Using a fluorescent second antibody and a nuclear stain (DAPI), the cells were analysed on a confocal microscope (Figure 3C). Using DAPI as reference, 200 cells were counted in each culture. In HEEC cultures 95 % of cells showed positive staining for CK18. In HESC cultures 97 % of cells were stained by vimentin and less than 1% for CK18. Using IHC, Norwitz et al. (1991) showed co-staining of cytokeratin 18 and vimentin in endometrial gland cells during the whole menstrual cycle. In our HEEC samples, less than 1 % of cells showed vimentin expression.

Therefore, a simple method to achieve highly purified HEEC and HESC cultures can be established from mid-secretory endometrial phase.

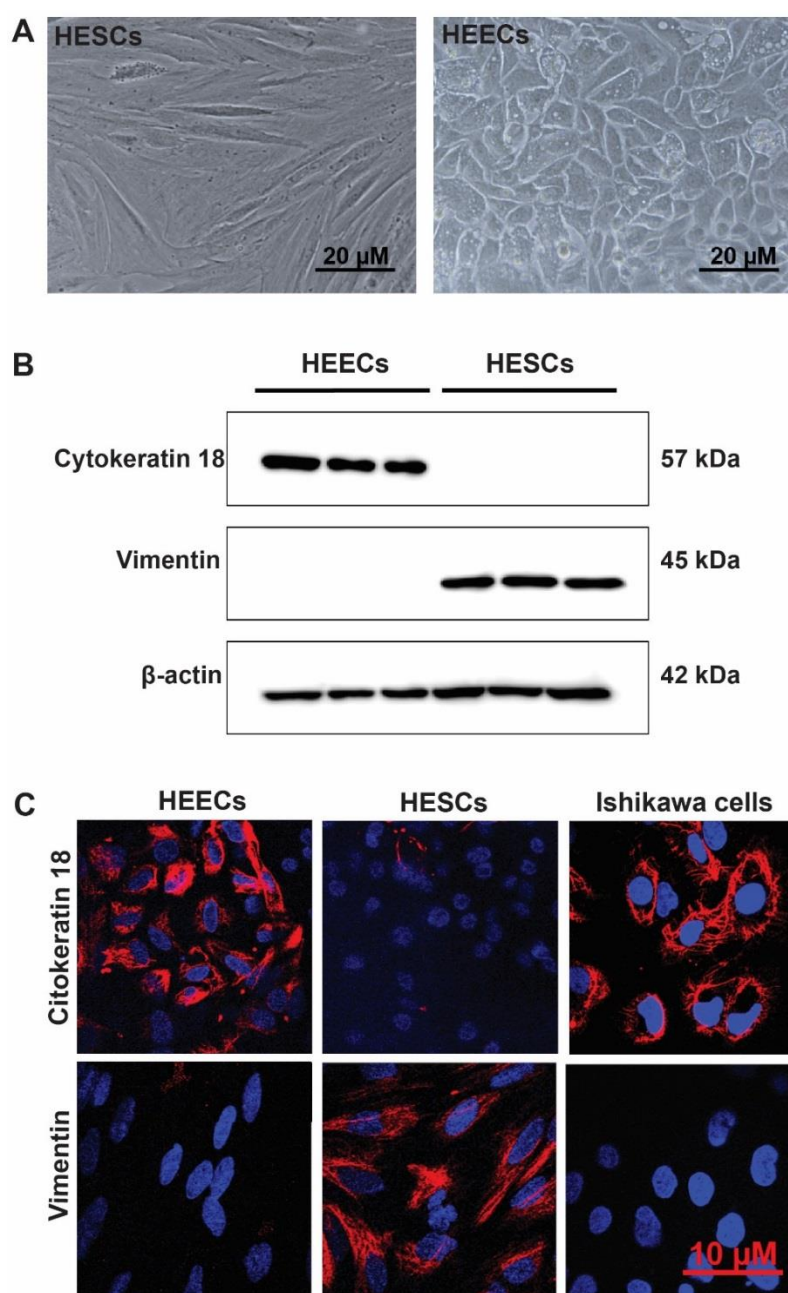


Figure 3.1 Characterization of purified cultures of primary HEECs and HESCs isolated from secretory endometrium. Paired HESC and HESC cultures from 3 patients were propagated for 7 days. **(A)** Bright field microscopy images of confluent HEECs and HESCs cultures **(B)** Western-blot analysis of CK18 and vimentin in cell lysates of paired HEECs. **(C)** Immunofluorescence of CK18 and vimentin in cultured HEECs, HESCs and Ishikawa cells (epithelial cell positive control) counterstained with DAPI. Less than 1 % of HEEC were vimentin positive (not shown).

3.2.2 Responses of HEECs to Trypsin

Trypsin-like serine proteases are released by the embryo and are known to have a role in blastocyst hatching (Sharma et al., 2006). It has been suggested that trypsin is an important molecule used by the embryo to signal to the uterine epithelium. In mice, this enzyme triggers decidualization through activation of epithelium sodium channels in luminal cells, leading to secretion of PGE₂ (Ruan et al., 2012). In humans, it was suggested that embryo-derived trypsin could be used by the HEECs for maternal recognition of the embryo quality (Brosens et al., 2014). To investigate whether trypsin induces secretion of PGE₂ in HEECs, I first treated several cultures with trypsin in a single pulse lasting 10 minutes, harvested total RNA 1, 3, 6 and 12 hours later, and measured the relative expression of *PTGS2*. Three HEEC cultures were used for each time-point. No *PTGS2* induction was observed at any time-point (Figure 3.2A). Since it was not possible to establish the time after trypsin exposure when *PTGS2* would have its highest induction, I decided to measure PGE₂ secretion in tissue culture supernatant, collected 24 hours after trypsin treatment. Different trypsin doses were applied and heterogeneous responses were obtained. One culture showed the highest induction of PGE₂ secretion with 10 nM trypsin. Increased secretion was observed with 50 nM and 150 nM trypsin in a second culture. And no response was detected in a third culture (Figure 3.2B), suggesting that cultured HEEC show variable responses to trypsin exposure.

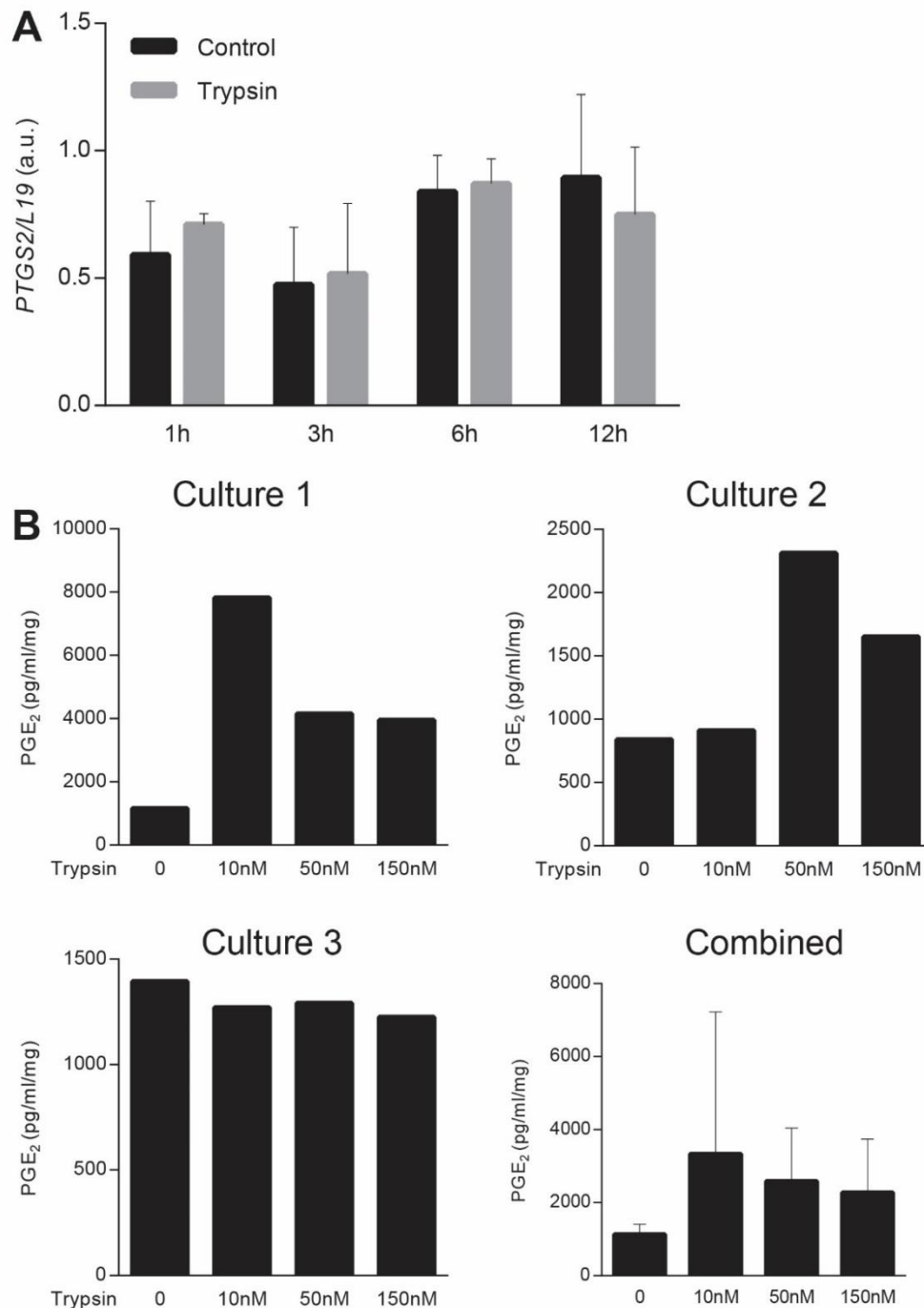


Figure 3.2 Variable responses of cultured HEECs treated with trypsin. (A) Normalized expression of *PTGS2* after the treatment with 10nM trypsin. mRNA was harvest at indicated time-points. Three HEEC samples were used for each time-point. Data are presented as mean \pm SD. $P > 0.05$. **(B)** PGE₂ secretion in culture supernatant of three HEEC samples, 24 hours after treatment with different doses of trypsin in a single pulse lasting 10 minutes. Data are presented as secretion normalized to total protein \pm SD. $P =$ non-significant (ns). P values were calculated using a non-parametric test (Friedman test).

3.2.3 *In vitro* HEECs Do Not Respond to Differentiation Cues

PAEP is the gene that encodes the different isoforms of glyodelin. Glyodelin-A is a marker of endometrial gland differentiation (Seppala et al., 2002). P4 induces glyodelin-A secretion by endometrial glands (Seppälä et al., 1987). hCG is also known to stimulate glyodelin-A (Uchida et. al., 2013). To test the effects of ovarian sex hormones and hCG in HEEC differentiation, I treated the cells with different combinations of E2, MPA and hCG, for 24 hours (Figure 3.3, top panel) and measured *PAEP* relative expression using RT-qPCR. No changes in *PAEP* expression were observed with any combination of hormones.

I also examined the effect of the same hormones on HEEC expression of *PTGS2* (Figure 3.3, bottom panel). No significant induction or inhibition of *PTGS2* expression was detected in HEECs treated with ovarian sex steroids or hCG, contradicting expected results. These findings suggest that HEECs change their behaviour *in vitro*, rendering them unresponsive to various differentiation stimuli.

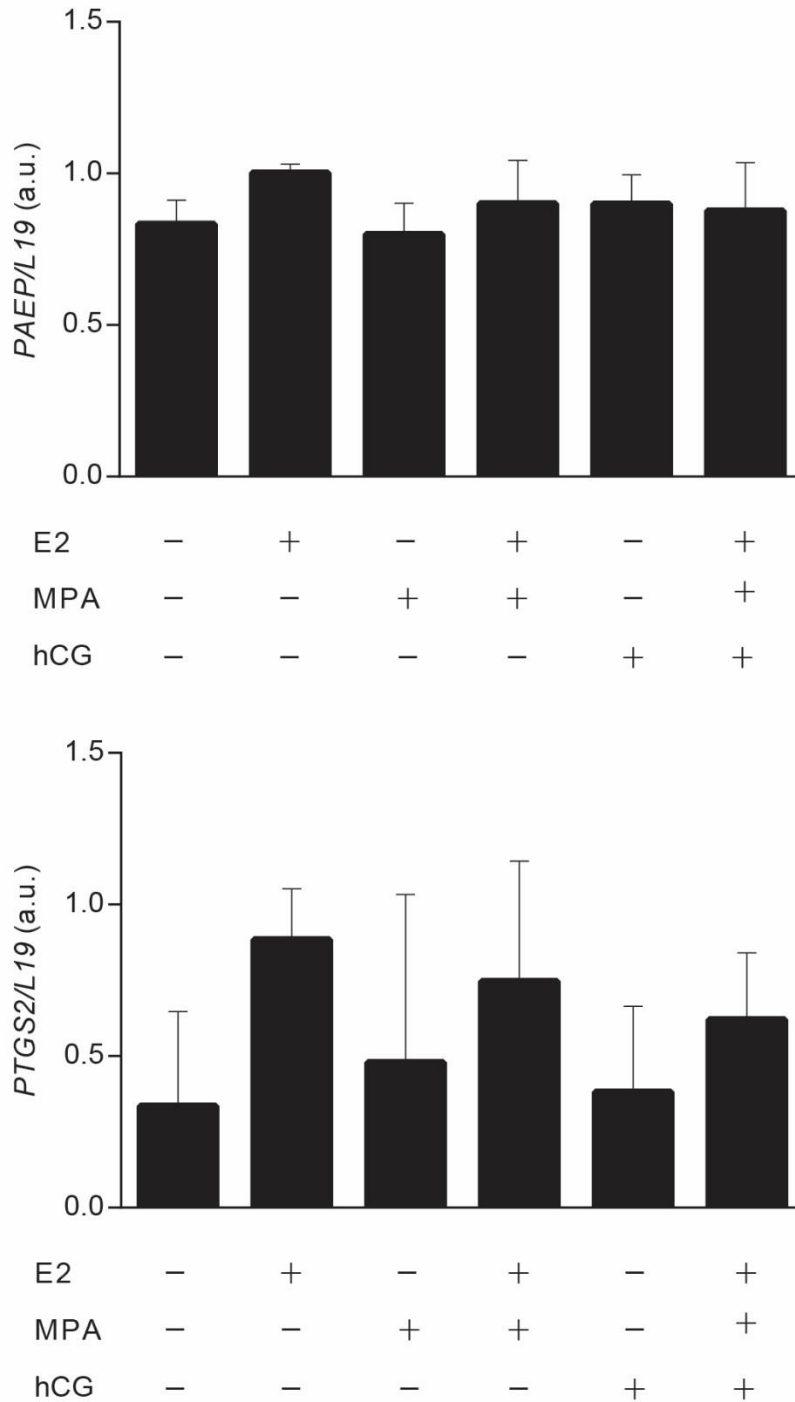


Figure 3.3 Unresponsiveness of cultured HEECs to differentiation cues. Normalized expression of *PAEP* and *PTGS2* in confluent HEECs treated with different combinations of 10 nM E2, 1 μ M MPA and/or 1 IU/ml hCG for 24 hours. Their presence or absence in the medium is shown by + or – in each column ($n = 3$). Data are presented as mean \pm SD. $P = ns$. P values were calculated using a non-parametric test (Friedman test).

3.2.4 Expression of Hormone Receptors and Protease Activated Targets in HEECs

Sex steroid hormones act through activation of their cognate nuclear receptors. hCG acts through its membrane bound G-protein coupled receptor, LHCGR. Trypsin cleaves epithelium sodium channels (ENaCs) on plasma membrane, increasing their open time probability (P_o). The enzyme also activates special G-protein couple receptors, named protease activated receptors (PAR), through cleavage. I interrogated if the variable responses of HEECs to ovarian steroids and trypsin could be explained by heterogeneity in the expression of related receptors and ion channels. To test this hypothesis, I harvested mRNA from 7 primary HEEC cultures and measured the expression of ER α (*ESR1*), progesterone receptor (*PGR*), hCG receptor (*LHCGR*), ENaC α , (*SCNN1A*), ENaC β (*SCNN1B*), ENaC γ (*SCNN1G*) and PAR2 (*F2RL1*), which is the main PAR activated by trypsin. Apart from PAR2, all the other receptors and the ion channels showed considerable variability in genomic expression between primary cultures (Figure 3.4A). To further investigate the variable effect of trypsin in HEECs, I performed fluorescent immunolocalization of ENaC α , β and γ in HEEC cultures, which revealed the staining confined to cells at the periphery of HEEC islands (Figure 3.4B). These data uncover a possible explanation for the heterogeneous response of HEEC when in monolayer culture.

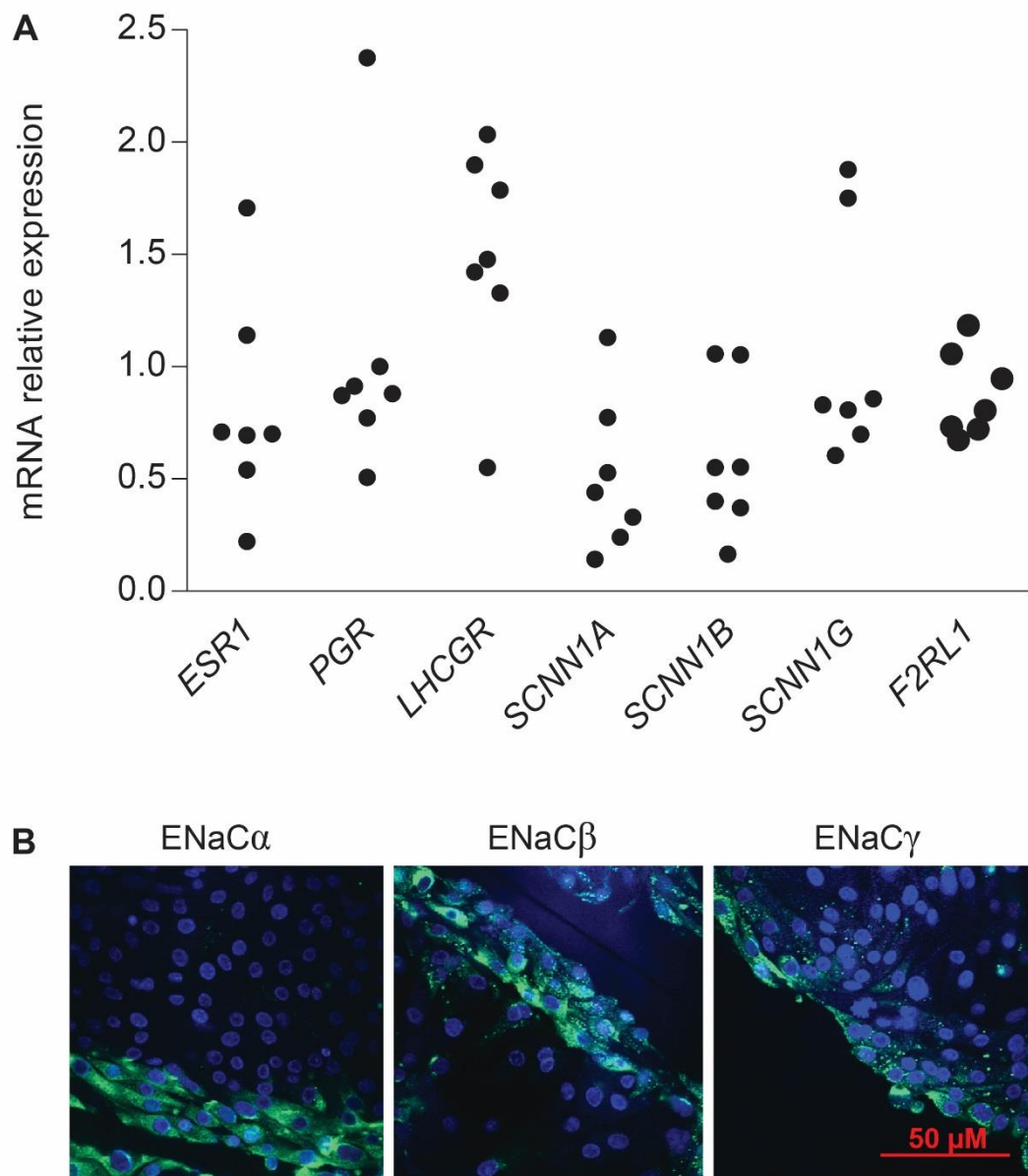


Figure 3.4 Heterogeneous expression of steroid hormone receptors and protease activated ion channels in HEECs. Seven HEEC cultures were grown for 8 days. **(A)** HEEC mRNA expression of selected genes. Results were normalized to the housekeeping gene (*L19*) expression. Data are presented as individual a.u. **(B)** Immunofluorescence of epithelial sodium channels HEEC cultures counterstained with DAPI (representative of $n = 7$).

3.2.5 Proliferation of Primary HEECs in Culture

HEECs soon lose their proliferative capacity when in culture, whereas HESCs can be easily cultured for longer periods (Kyo et al., 2003). E2 stimulates epithelial proliferation *in vivo*. By contrast P4 counteract the mitogenic effects of E2. Proliferative effects of E2 in HEECs are mediated by stromal ER α while inhibition of oestrogen-driven epithelial proliferation are mediated by epithelial and stromal PR (Vasquez and DeMayo, 2013). In order to investigate the growth rate of HEECs and HESCs, proliferation in purified cultures were monitored by an xCELLigence real-time cell analyser. HESCs continuously grew, maintaining their proliferative capacity for over 72 hours. By contrast, HEECs showed a highly restricted growth, with minute change after 72 hours (Figure 3.5A).

To determine the effects of E2 and P4 on proliferation of isolated *in vitro* HEECs, an xCELLigence real-time cell analyser was used for monitoring the growth of HEECs either untreated or treated with E2 or P4 or the combination of both. No effect on HEEC growth was detected over 60 hours (Figure 3.5B). This confirms that HEECs undergo growth arrest when cultured under standard conditions.

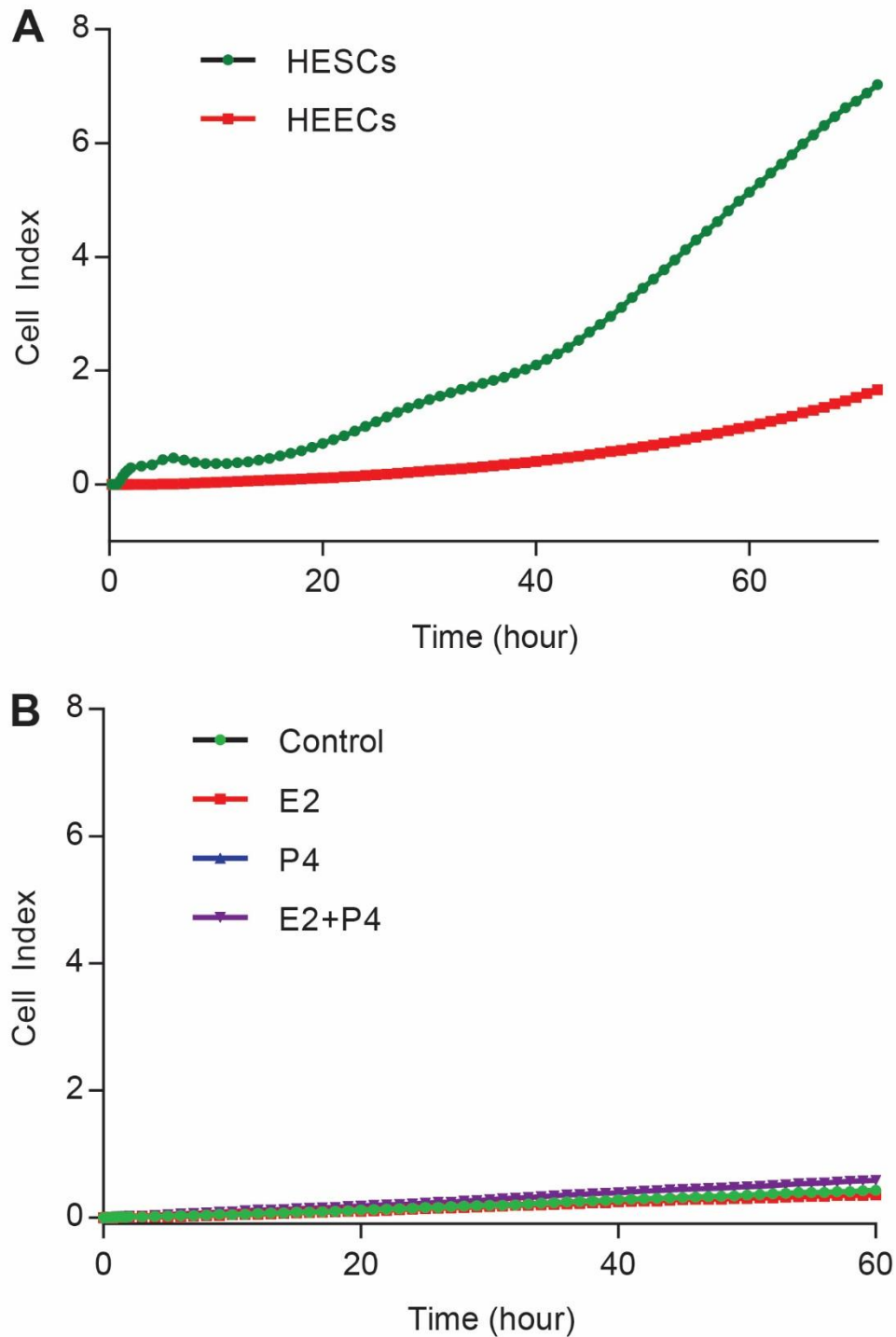


Figure 3.5 Growth arrest of HEECs in culture. Ten thousand cells were seeded into 0,2 cm² wells. **(A)** Real-time cell analysis of attachment and growth of HESCs ($n = 6$) and HEECs ($n = 6$) measured by electrical impedance, using an xCELLigence analyser for 72 hours. **(B)** Real-time cell analysis of attachment and growth of HEECs for 60 hours. The cells were either untreated, or treated with 10 nM E2 or 1 μ M P4 or the combination of both ($n = 3$).

3.2.6 Purified HEECs from Mid-Luteal Biopsies Exhibit Acute Senescence in Culture

HEECs derived from the secretory phase of the menstrual cycle develop senescence in culture after few days (Valentijn et. al., 2015). To corroborate this previous observation, I compared the senescence in paired HESCs and HEECs from mid-secretory phase. Cellular aging results in accumulation of toxic by-products and macromolecules in lysosomes. The activity of β -galactosidase reflects increased lysosomal mass (Matjusaitis et al., 2016). Using senescence associated β -galactosidase (SA- β -gal) staining, it was possible to detect marked presence of the senescence dye in HEECs, whereas few spots were observed in HESCs (Figure 3.6A). SA- β -gal activity was also compared between the two cell types, using a fluorometric assay. Activity in HESCs was approximately 80 % lower than in HEECs (Figure 3.6D). Using Western-blot analysis of senescence markers (Binet et. al., 2009, Freund et al., 2012, Guerreiro and Gil, 2016) in HESCs and HEECs, I demonstrated a typical profile of senescence in HEECs, exemplified by higher expression of P53, P16 and histone H3, and lower expression of lamin B1 and high mobility group box family member 2 (HMGB2) compared to HESCs (Figure 3.6B). Immunohistochemistry of mid-luteal endometrial biopsy (LH+9) demonstrated predominance of P16-positive cells in both GE and LE compared to stroma (Figure 3.6C). Taken together the data corroborate the observation that HEECs from mid-secretory phase develop acute senescence when isolated and cultured.

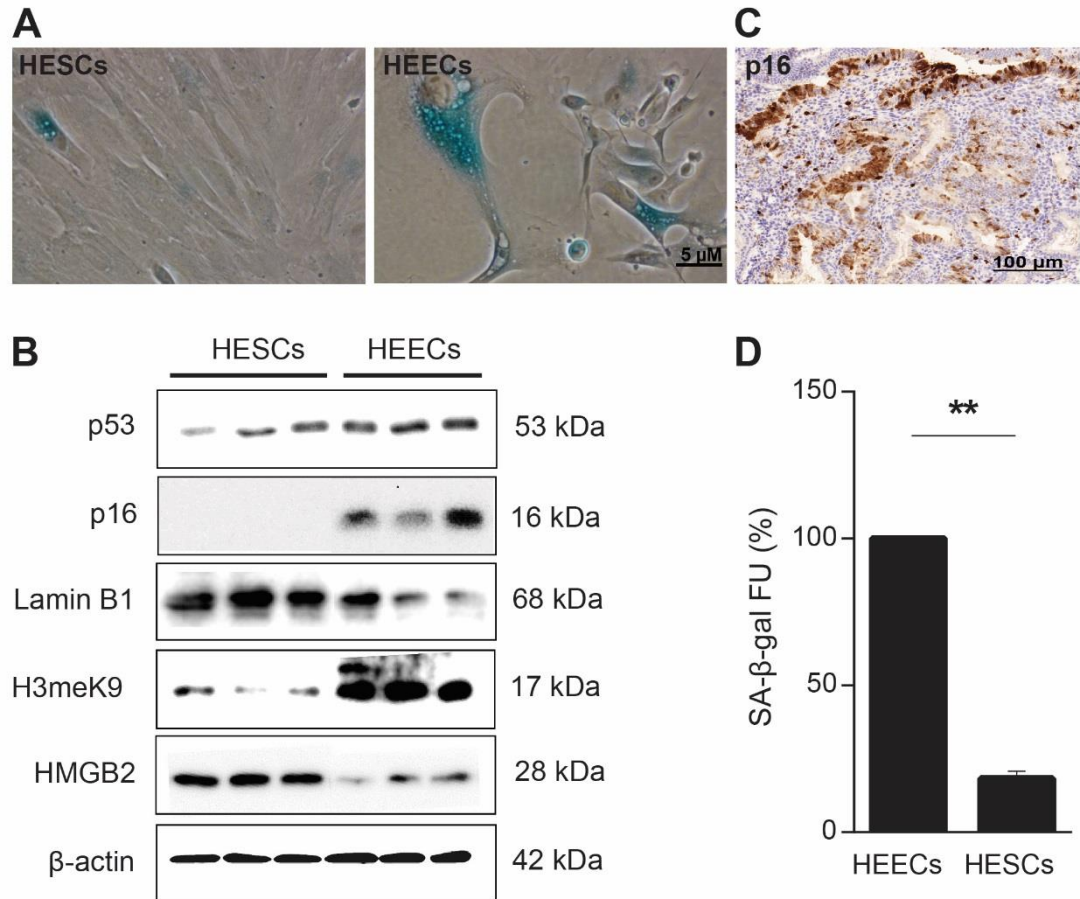


Figure 3.6 Acute senescence of cultured HEECs derived from the secretory phase of the menstrual cycle compared to HESCs from the same period. (A) SA- β -gal staining of paired HESCs and HEECs cultured for 9 days (representative of $n = 3$). **(B)** Western-blot analysis of senescence markers in three paired HESC and HEEC samples in culture for 7 days **(C)** IHC of Mid-secretory human endometrium stained for P16 (representative of $n = 3$). **(D)** SA- β -gal activity in HEECs ($n = 9$) and HESCs ($n = 9$) cultured for 7 days, measured by fluorometric assay. Data are presented as percentage of reduction of SA- β -gal activity in relation to HEEC activity \pm SD. ** $P = 0.007$. P values were calculated using a non-parametric test (Wilcoxon signed-rank test).

3.2.7 Conditional Reprogramming of Primary HEECs Using an Irradiated Feeder Layer and a Rho-Associated Kinase (ROCK) Inhibitor

In an attempt to reverse the senescence-associated cell cycle arrest of HEECs in culture, I used a combination of a feeder layer of irradiated 3T3 swiss albino mouse embryo fibroblasts (3T3 SAFs) and a ROCK inhibitor (Y-27632) to conditionally reprogramme HEECs. This method has been employed for keratinocytes and non-keratinocyte epithelial cells (prostate cells and mammary cells) (Liu *et al.*, 2012), but not for HEECs. In previous studies, the feeder layers were gamma-irradiated for growth arrest (Liu *et al.*, 2012, Yuan *et al.*, 2012). Due to the unavailability of a suitable ionizing radiation source and the requirement of a dedicated tissue culture room to work with radioactive cells, I opted for irradiating the 3T3 SAFs with ultraviolet C light (UVC). This short wave ultraviolet light (254 nm), at doses above 50 J / m², triggers DNA damage, cell cycle arrest and apoptosis (Gentile *et al.*, 2003). Some studies have suggested that apart from inducing growth arrest, irradiation of feeder layer increases the release of soluble factors that are crucial for cell reprogramming (Li *et al.* 2010, Palechor-Ceron *et al.*, 2013). 3T3 cells were cultured until 70 % confluence, and were irradiated with 100 J / m² dose of UVC. HEECs were seeded on top of the feeder layer, and cultured with medium supplemented with 7.5 uM / l Y-27632 (CRC medium) (Figure 3.7A). Once confluent, cultures were differentially trypsinized and passaged onto fresh dishes. The technique is based on the differences in cell attachment. Fibroblasts from feeder layer detach first, whereas HEECs are more tightly adherent, thus taking longer in trypsin-EDTA solution to dissociate (see 'Methods'). A frequently faced problem with these cultures is contamination of the passaged reprogrammed HEECs with fibroblasts from the feeder layer (Figure 3.7B). Despite most of the 3T3 SAFs being eliminated with the

differential trypsinization, the presence of few viable cells resulted in overgrowth of the feeder layer at the expense of the HEECs.

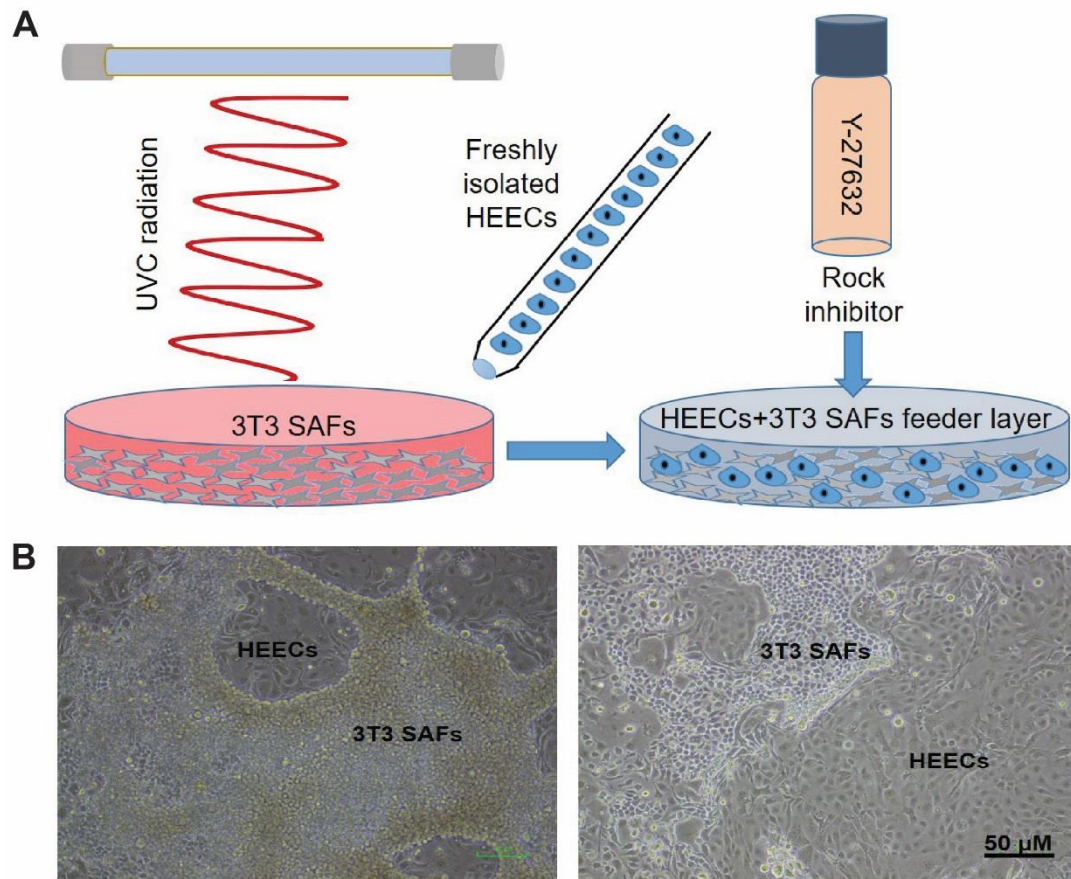


Figure 3.7 Conditional reprogramming of HEECs using a feeder layer of irradiated 3T3 SAFs and a ROCK inhibitor. (A) Cartoon representing the use of a 3T3 SAF feeder layer in combination with a ROCK inhibitor (Y-27632), for conditional reprogramming of HEECs. **(B)** Culture of conditionally reprogrammed HEECs with a feeder layer of irradiated 3T3 SAFs and 7.5 μM / l Y-27632 at P0 (left panel), and conditionally reprogrammed HEECs at P1 for 7 days after the differential trypsinization to remove the feeder layer, exhibiting contamination with 3T3 SAFs (right panel).

3.2.8 Conditioned Medium from UVC Irradiated 3T3 SAF Substitutes for Feeder Layers in the Conditional Reprogramming of HEECs

Due to the contamination of HEECs with cells from the feeder layer and the difficulty of synchronising the irradiation of 3T3 SAFs with the isolation of HEECs, I explored a feeder-free method for conditional reprogramming of primary cultures. It has been demonstrated that soluble factors in the conditioned medium from irradiated 3T3 SAFs could substitute for feeder layers in conditional reprogramming of epithelial cells (Palechor-Ceron et al., 2013). The same authors showed that media from irradiated 3T3 SAF was most effective when collected 48-72 hour after the irradiation. This period correlated with apoptosis of feeder layers, as demonstrated by caspase 3 and 7 activity. Using conditioned medium from irradiated 3T3 SAF (CM_i), in combination with a ROCK inhibitor, I developed a method for conditionally reprogram HEECs without the requisite of feeder layers. Seventy percent confluent 3T3 SAFs were irradiated with a 100 J/m² UVC dose, and culture supernatants were harvested 3 days later. CM_i was mixed with CRC medium in a 1/4 ratio and the resulting mixture (final CRC) was used as culture medium for HEECs (Figure 3.8A). The resulting cultures were pure and morphologically indistinguishable from un-reprogrammed HEECs cultures (Figure 3.8B). To test whether the reprogrammed cells retained their epithelial phenotype, and to demonstrate the purity, three HEEC cultures were immunostained for CK18 and vimentin (Figure 3.8D). More than 97% of cells were positive for CK18, whereas less than 3% were vimentin positive. Next, I tested the proliferation capacity of reprogrammed HEECs using xCELLigence real-time cell monitoring. The use of CM_i or ROCK inhibitor moderately increased proliferation of HEECs, but the combination of both considerably improved growth (Figure 3.8C). These findings demonstrate that conditional reprogramming of HEECs can be

successfully achieved without ionizing radiation or the need of co-culturing with a feeder layer.

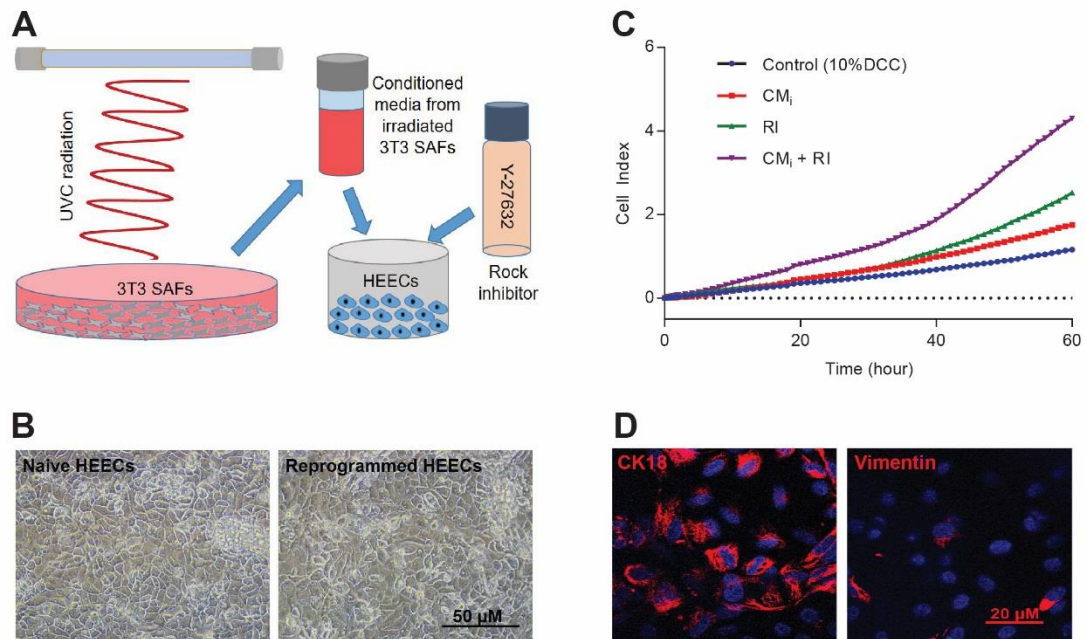


Figure 3.8 Conditional reprogramming of HEECs using conditioned medium from irradiated 3T3 SAFs and a ROCK inhibitor. (A) Cartoon showing the conditional reprogramming of HEECs using CM_i in combination with a RI. **(B)** Bright field images of confluent cultures of paired naive HEECs and conditionally reprogrammed HEECs. **(C)** Real-time cell xCELLigence analysis of attachment and growth of HEECs. The cells were either untreated, or treated with CM_i or 10 μ M/l ROCK inhibitor or the combination of both, for 60 hours ($n = 4$) **(D)** Immunofluorescence of CK18 and vimentin in conditionally reprogrammed HEECs cultured for 7 days, counterstained with DAPI.

3.2.9 Impact of Conditional Reprogramming in Expression of Hormone Receptors, Protease Activated Targets and *PAEP*

Cell reprogramming can affect cell phenotype of the resulting derivatives (Supryniewicz et al., 2012). In order to test if conditional reprogramming with CM_i and ROCK inhibitor changes the phenotype of HEECs, and the reversibility of this effect, I measured mRNA expression of trypsin-activated ion channels, PAR2 (*F2RL1*), ER α , PR and *PAEP* in naive and reprogrammed HEECs; and upon reprogramming withdrawal. One group of cells was kept in standard culture conditions for 5 days. Another group was conditionally reprogrammed for 5 days. A third group was initially reprogrammed for 5 days, followed by reprogramming withdrawal for 48 hours. Except for *LHCGR* and *F2RL1*, all the other genes were up-regulated upon conditional reprogramming (Figure 3.9). This may suggest that the cells were more differentiated. More important was the fact that reprogramming withdrawal restored the basal phenotype of HEECs, proving that it is possible to transiently induce proliferation. Arguably, it may also be possible that the reprogramming phenotype is closer to the one observed *in vivo*.

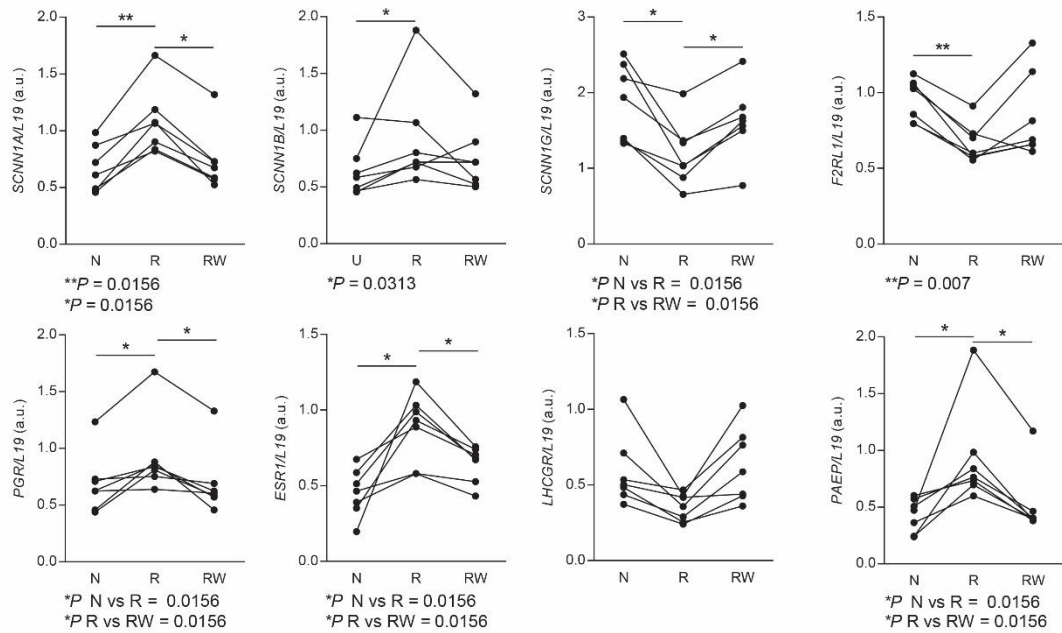


Figure 3.9 Expression of protease activated targets, hormone receptors and PAEP in conditionally reprogrammed HEECs. Paired HEECs were either kept in standard culture for 5 days (N), or were conditionally reprogrammed (R) for 5 days, or were reprogrammed for 5 days, followed by reprogramming withdrawal for 48 hours (RW). Data are presented as individual a.u. ($n = 7$). P values were calculated using a non-parametric test (Friedman test).

3.2.10 Reversal of Cellular Senescence upon Conditional Reprogramming of HEECs

To determine the effect of conditional reprogramming on HEEC senescence, I measured cellular senescence by SA- β -gal staining and activity. Naive HEECs exhibited more intense SA- β -gal staining after 11 days in culture when compared to reprogrammed HEECs (Figure 3.10A). For the SA- β -gal activity, HEECs were maintained for 5 days in standard culture, then conditionally reprogrammed for 5 days, or reprogrammed for 5 days followed by reprogramming withdrawal for 48 hours. SA- β -gal activity was approximately 50 % lower in reprogrammed HEECs, but was restored upon withdrawal of CM_i and ROCK inhibitor (Figure 3.10B). Loss of telomerase activity is the main feature of cellular replicative senescence (Campisi and Fagagna, 2007). To test if this enzyme is affected by conditional reprogramming, I measured the mRNA expression of reverse transcriptase telomerase (*TERT*) in cells maintained in standard culture for 5 days, in cells reprogrammed for 5 days, and upon withdrawal of the reprogramming cues for 48 hours. A 376 % increase in *TERT* expression was observed, with return to basal levels upon reprogramming withdrawal (Figure 3.10D). To further corroborate the findings, I analysed the expression of senescence markers in naive and reprogrammed HEECs using Western-blot (Binet et. al. 2009, Freund et al., 2012, Guerreiro and Gil, 2016). Decreased expression of P53, P16 and histone H3 in reprogrammed HEECs was observed along with an increase in lamin B1 and HMGB2 expression, which indicates reduction in senescence upon reprogramming (Figure 3.10C).

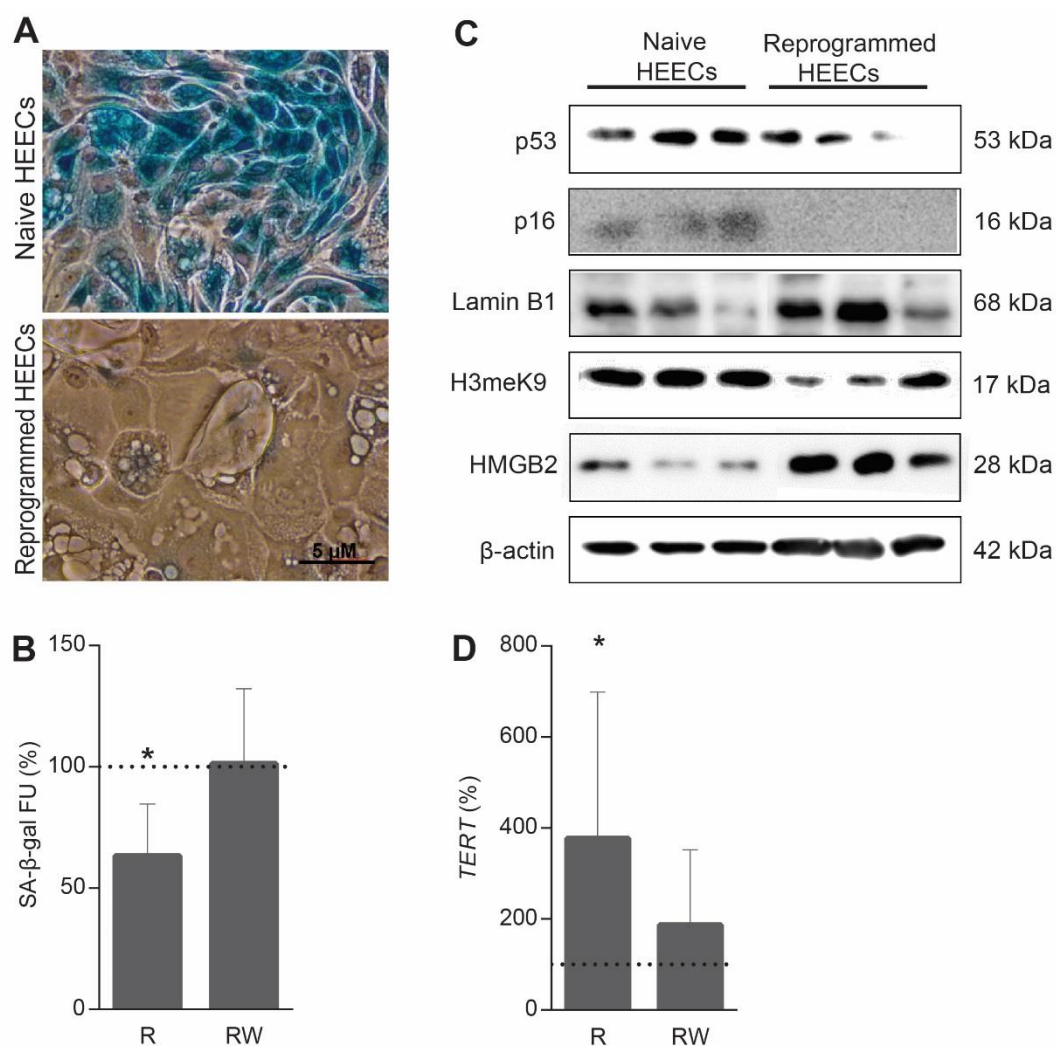


Figure 3.10 Reversal of HEEC senescence upon conditional reprogramming.

(A) SA-β-gal staining of paired un-reprogrammed and conditionally reprogrammed HEECs in culture for 11 days. **(B)** SA-β-gal activity of paired naive HEECs, conditionally reprogrammed HEECs (R), and of HEECs in which conditional reprogramming was withdrawn (RW). Dotted line indicates SA-β-gal activity in naive HEECs. Data are presented as percentage of increase/decrease of SA-β-gal activity in relation to the activity in naive HEECs \pm SD ($n = 3$). $*P = 0.013$. **(C)** Western-blot analysis of senescence markers in paired conditionally reprogrammed and naive HEECs from three HEEC samples. **(D)** Normalized HEEC expression of *TERT* in paired naive, R and RW HEECs. Dotted line indicates mRNA expression of naive HEECs. Data are presented as percentage of induction/inhibition in relation to the naive HEEC mRNA expression \pm SD ($n = 7$) $*P = 0.03$. P values were calculated using a non-parametric test (Friedman test).

3.2.11 Conditionally Reprogrammed HEECs Remain Unresponsive to Differentiation Cues or Trypsin

My data suggested that reprogrammed HEECs acquire a more differentiated phenotype, with higher expression of ovarian steroid receptors. Therefore, I decided to investigate the effect of E2 and P4 on reprogrammed HEEC proliferation. The growth and attachment of these cells were monitored through real-time cell analysis using an xCELLigence system (Figure 3.11A), and final CRC medium was supplemented with E2, P4 or the combination of both. No effect on HEEC proliferation was detected indicating that reprogrammed HEECs, like their naive counterparts (Figure 3.5B) are not responsive to either E2 or P4. Next I tested if the expression of two differentiation markers, *PAEP* and *PTGS2*, could be induced in reprogrammed cells treated with E2, MPA or hCG alone or in combination. No significant changes in *PAEP* or *PTGS2* (Figure 3.11B) were observed. I have demonstrated that mRNA expression of the three ENaC variants were also up-regulated upon reprogramming. To test if this remodelling would bestow the cells the ability to respond to serine proteases, I treated reprogrammed HEECs with trypsin in a single pulse lasting 10 minutes, and harvested the culture supernatant 24 hours later. No increment in PGE₂ secretion into the culture medium was observed (Figure 3.11C). These data demonstrated that conditional reprogramming does not modify the capacity of HEECs to respond to differentiation cues from the ovary or the implanting blastocyst.

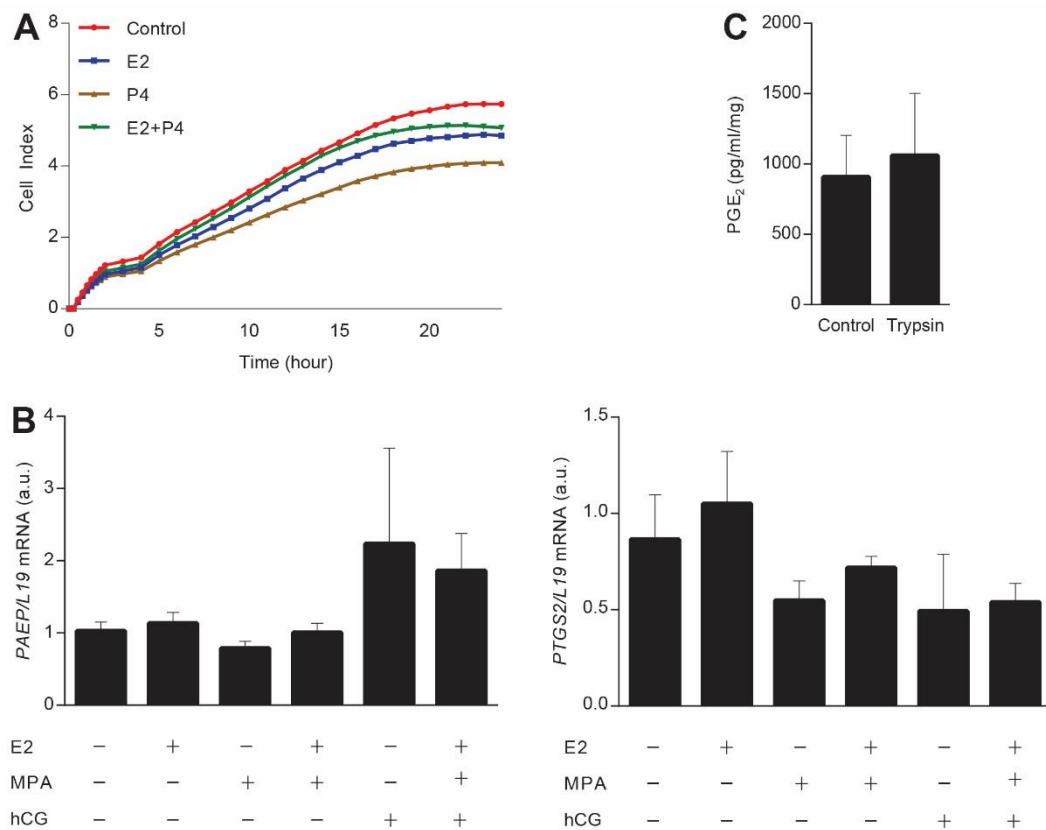


Figure 3.11 Unresponsiveness of conditionally reprogrammed HEECs to differentiation cues and embryonic signals. (A) 10^5 cells / well were seeded. Real-time xCELLigence monitoring of conditionally reprogrammed HEEC proliferation. The cells were either untreated (Control), or treated with 10 nM E2 or 1 μ M P4 or the combination of both, for 24 hours ($n = 3$). **(B)** Normalized expression of *PAEP* and *PTGS2* in reprogrammed HEECs treated with different combinations of 10 nM E2, 1 μ M MPA and 1 UI/ml hCG, for 24 hours ($n = 3$). Their presence or absence in the medium is shown by + or – in each column. Data are presented as mean \pm SD. $P =$ ns. P values were calculated using a non-parametric test (Friedman test). **(C)** Secretion of PGE₂ in reprogrammed HEEC culture supernatant, 24 hours after a single 10 nM trypsin pulse lasting 10 minutes. Data are presented as mean secretion normalized to total protein in cell lysates \pm SD ($n = 3$) $P =$ ns. P values were calculated using a non-parametric test (Wilcoxon signed-rank test).

3.2.12 HEEC Differentiation in Response to Conditioned Medium from Decidualized HESCs

A fine balanced cross-talk between the epithelial and the stromal compartments of the endometrium is crucial to create a receptive environment for the implanting embryo (Pawar et al., 2014). Paracrine factors regulated by oestrogen and P4 mediate this dialogue. For example, the oestrogen-driven proliferation of the epithelial cells is mediated by stromal ER (Cooke et al., 1997) and anti-mitogenic effects of progesterone in the epithelial compartment are regulated by PR in the stromal cells (Hantak et al., 2014). Furthermore, factors like Wnt5a, Wnt4, HOXA10 and HOXA11, expressed by the endometrial stromal compartment, are critical for development of endometrial glands (Mericskay et al., 2004, Zhang and Yan, 2015). The highest expressions of gland differentiation markers such as glycodeclin and dipeptidyl peptidase-4 (DPP4) are observed during the mid-secretory phase, concurring with the highest levels of P4 and the stromal decidualization (Imai et al., 1992, Seppälä et al., 2002). Gland differentiation could be induced directly by P4, which was not verified in my previous experiments (Figure 3.3A and figure 3.11C), or it could be indirectly triggered by soluble factors derived from the decidualized stroma. This phenomenon has been already demonstrated. In other species, PRL, which is an important protein expressed by the stroma upon decidualization, can induce uteroglobin expression in endometrial glands (Spencer, 2014). Uteroglobin, encoded by *SCGB1A1*, is mostly secreted in the uterine lumen during the receptive phase in human uterus (Müller-Schöttle et al., 1999).

To investigate the effect of decidualized HESCs in gland differentiation, I used conditioned media from pooled HESCs from 5 biopsies. The supernatant was collected from undifferentiated HESCs and from cells decidualized with cAMP and MPA for either 4 or 10 days. The medium was refreshed 24 hours before collection, to remove cAMP and MPA. Confluent cultures of reprogrammed HEECs were treated

with unconditioned medium and with conditioned medium from decidualized and undecidualized HESCs. mRNA was harvested 24 hours later (Figure 3.12A). Significant up-regulation of *PAEP* was observed with conditioned medium from HESCs decidualized for 4 and 10 days. (Figure 3.12B, left). *PTGS2* expression in HEECs was also measured to investigate whether the decidualized HESCs released factors that further enhance epithelial differentiation in a paracrine manner. No response was detected when compared to HEECs cultured in standard medium (Figure 3.12B, right).

Since PRL is a major secretory product of human decidua, I speculated if this protein was the effector of the previous results observed in the HEECs treated with the decidualized HESC conditioned medium. To test this hypothesis, I treated HEEC cultures with 20 ng / ml PRL for 24 hours and measured *PAEP* expression. However, the treatment did not induce any response (Figure 3.12C).

These observations suggest that soluble factors released by the decidualized endometrial stromal compartment induce differentiation in uterine glands. The factor(s) responsible for this effect are undetermined.

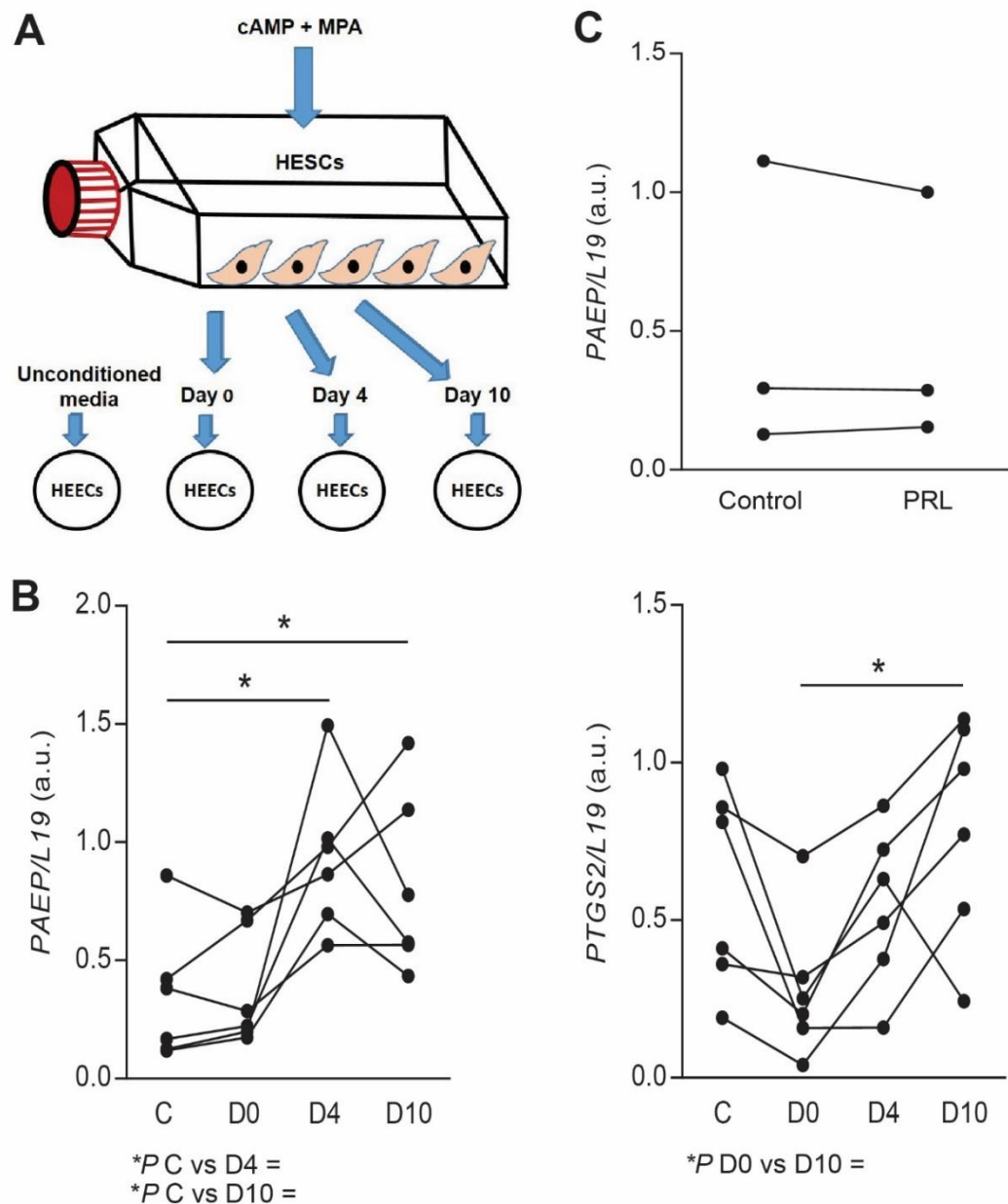


Figure 3.12 HEEC differentiation by soluble cues from decidualized HESCs. (A) Cartoon showing HESC decidualization with 0.5 mM cAMP and 1 μ M MPA, and the retrieval of conditioned medium after 0, 4 and 10 days of treatment. The medium was refreshed 24 hours before the supernatant harvesting to remove cAMP and MPA. **(B)** Normalized expression of *PAEP* and *PTGS2* in HEECs that were either untreated (C), or treated for 24 hours with medium from undecidualized HESC (D0), or HESC decidualized for 4 (D4) or 10 (D10) days ($n = 6$). P values were calculated using a non-parametric test (Friedman test). **(C)** Normalized expression of *PAEP* in HEECs treated with 20 ng / ml PRL for 24 hours. Data are presented as individual a.u. ($n = 3$). P values were calculated using a non-parametric test (Wilcoxon signed-rank test).

3.2.13 Induction of HEEC Differentiation and HESC Decidualization upon Co-Culture of the Two Cell Types

To further validate the observations that the endometrial stromal compartment induced glandular differentiation in HEECs, I established a co-culture system of HESCs and HEECs, using transwell inserts with permeable porous membranes (Figure 3.13A, top). A pool of HESCs from 5 different patients was cultured in 24-well plates and decidualized with cAMP and MPA. Reprogrammed HEECs were cultured on the membrane of transwell inserts in separate wells until confluence was reached. After 4 days of HESC decidualization, cAMP and MPA were withdrawn, and the inserts with HEECs were placed into the wells with decidualized HESC for 24 hours. mRNA from HEECs was harvested, and subjected to RT-qPCR. Up-regulation of *PAEP* was observed when compared to non-cocultured HEECs (Figure 3.13C).

The presence of the endometrial glands has been proven essential for decidualization. In mice lacking uterine glands, decidualization is totally abolished and implantation does not occur (Lejeune *et al.*, 1981, Filant & Spencer, 2013). Some factors expressed by endometrial glands have been suggested as potential candidates for this stromal decidualization role. *Lif* and *Spink3* are the most promising (Cheng *et al.*, 2000; Cheng *et al.*, 2010). However, the dependency of decidualization on uterine glands in humans has not been demonstrated yet. To investigate if HEECs have any impact on stromal decidualization, I applied the same co-culture system. The pool of HESCs was seeded in 24-well plates, and HEECs from three different endometrial biopsies were cultured in transwell inserts. HESCs were cultured in 2 % DCC medium, for 8 days, with or without HEEC inserts (Figure 3.13A, bottom). mRNA from HESC was harvested, and *PRL* was measured. A significant induction of *PRL* in HESCs co-cultured with HEECs (S/E) was detected when compared to HESCs in monocultures (S) (Figure 3.13D).

These data confirm the influence of decidualized HESCs in endometrial gland differentiation and suggest that, as is the case in mice, signals emanating from glands, such as PGE_2 , contribute to differentiation of HESCs into decidual cells.

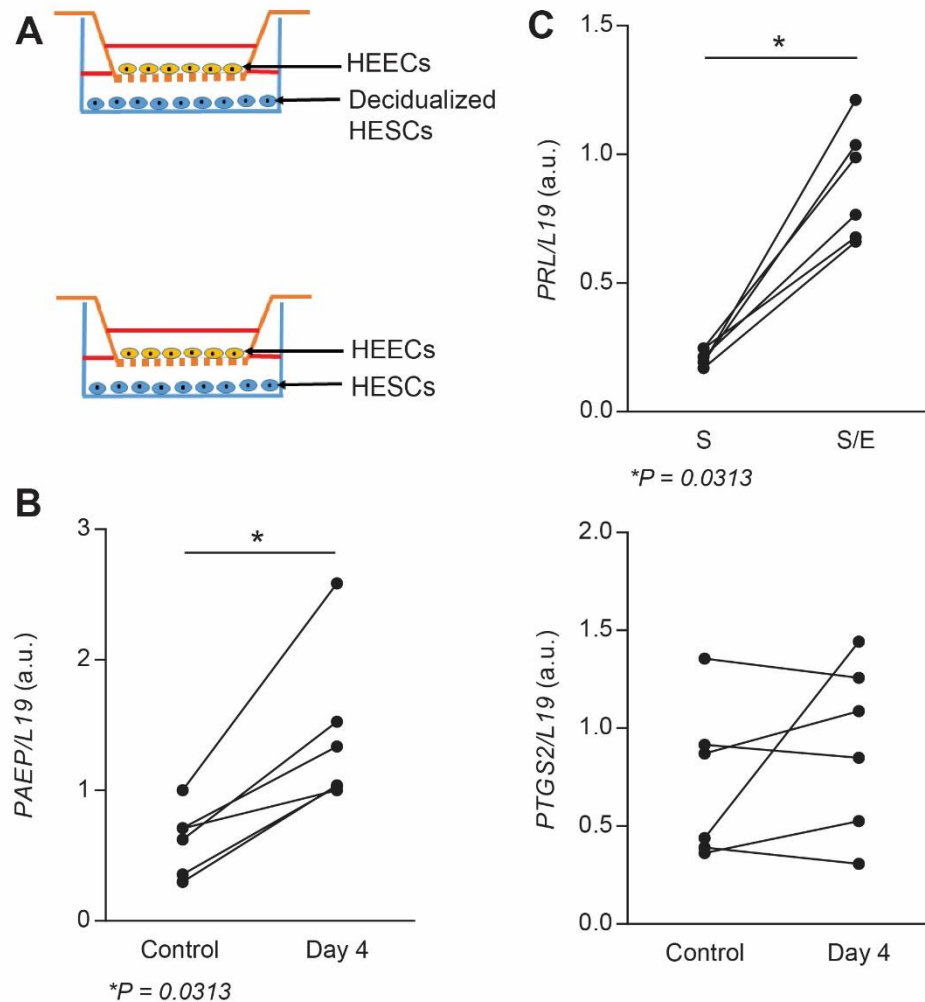


Figure 3.13 Induction of differentiation in HEECs and decidualization in HESCs when the two cell types are co-cultured. (A) Cartoon showing the system for co-culture of HEECs with decidualized HESCs, using transwell inserts with microporous membrane (top) and the system for co-culturing HEECs and HESCs (bottom). **(B)** Normalized expression of *PAEP* and *PTGS2* in HEECs co-cultured for 24 hours with decidualized HESCs ($n = 6$). Transwell inserts with confluent reprogrammed HEECs were placed into the wells with HESCs decidualized for 4 days, and were co-cultured for 24 hours in 2% DCC. **(C)** Normalized expression of *PRL* in HESCs after 8 days in co-culture with HEECs. Data are presented as individual a.u. ($n = 6$). P values were calculated with a non-parametric test (Wilcoxon signed rank test).

3.2.14 Proliferation and Partial Reversal of Senescence in HEECs Exposed to Conditioned Medium from Decidualized HESC

As aforementioned, raising levels of P4 during the luteal phase inhibit the mitogenic effects of E2 in the epithelial compartment (Pierro et al., 2001, Chen et al., 2005). At the same time, senescence of HEECs peaks at mid-secretory phase, coinciding with the highest serum concentrations of P4 (Williams et al., 2001). Endometrial gland function must be safeguarded to provide histiotrophic support to the implanting blastocyst. I examined if decidualized HESCs, through secretion of soluble factors, could rescue the gland cells from growth arrest and acute senescence.

Using xCELLigence real-time cell monitoring, I analysed the effects of conditioned medium from HESC decidualized for 4 days, ROCK inhibitor, and the combination of both in HEEC proliferation. HEEC growth was discretely enhanced with the use of conditioned medium from decidual HESCs. Proliferation of HEECs was even further increased with the addition of ROCK inhibitor (Figure 3.14A).

As previously discussed, senescence in the endometrial epithelial compartment peaks at mid-secretory phase, coincident with the highest levels of P4. To test if P4 affects the senescence in HEECs in an indirect paracrine way through inducible soluble factors from stromal compartment, I treated HEECs for 24 hours with conditioned medium from HESCs decidualized for 4 days and measured SA- β -gal activity. Strikingly, a mean reduction of 24% was observed (3.14B).

This suggests that decidualized HESCs induce proliferation and partially reverse senescence in HEECs.

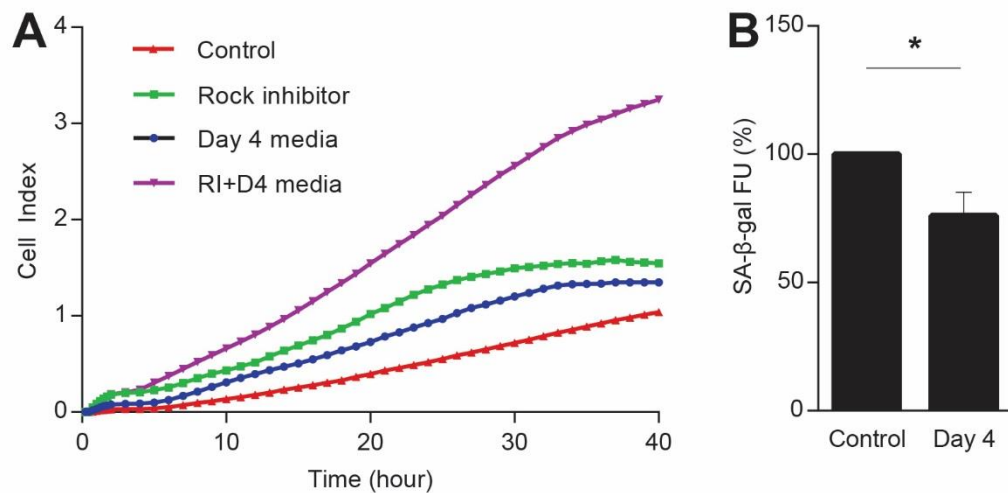


Figure 3.14 Proliferation and reversal of senescence in HEECs treated with conditioned medium from decidualized HESCs. (A) Real-time xCELLigence analysis of HEEC attachment and growth for 40 hours. HEECs were either untreated (control), or treated with 7.5 μ M Y-27632 (RI) or conditioned medium from HESCs decidualized for 4 days or the combination of both ($n = 4$). **(B)** SA- β -gal activity in HEECs either untreated (control) or treated for 24 hours with conditioned medium from HESCs decidualized for 4 days. Data are presented as percentage of reduction of SA- β -gal activity in relation to the control \pm SD ($n = 4$). * $P = 0.03$. P values were calculated using a non-parametric test (Wilcoxon signed-rank test).

3.3 Discussion

Isolation and culture of HEECs are pivotal for understanding the implantation process and its disruptions. Primary HEECs are hard to culture, and the problem is further exacerbated when they derive from mid-secretory phase endometrium. One possible explanation for this refractoriness is the proportion of progenitor, transit amplifying and terminally differentiated cells in the tissue. Chan et al. (2004) demonstrated that 0.22 % of HEEC population was comprised of progenitor cells in contrast with 1.25 % of HESC. The cells were isolated from hysterectomy specimens, which included the basal and the functional layers of the endometrium. It has been suggested that the majority of the epithelial progenitor cells reside in the gland segments of basalis (Nguyen et al., 2012, Valentijn et al., 2013). Therefore it is reasonable to postulate that the percentage of human endometrial epithelial progenitor cells would be even lower in Endocell Sampler biopsies.

Conditional reprogramming rescued the cells from acute senescence and improved *in vitro* proliferation. The exact mechanism by which this was achieved is yet to be uncovered, although many explanations can be inferred from the current knowledge. The effect of irradiated feeder layers and their soluble factors on telomerase activity has been demonstrated (Liu et al., 2012, Palechor-Ceron et al., 2013). The up-regulated expression of *TERT* upon reprogramming was observed in my study (Figure 3.10C). Chapman et al. (2010) observed an increased expression of avian myelocytomatosis viral oncogene homolog (MYC) in human foreskin keratinocytes treated with ROCK inhibitor. MYC induces transcription of TERT through promoter *e*-boxes. Yu et al. (2012) verified that a ROCK inhibitor increased cyclin-dependent kinase 4 (CDK4) and Cyclin D1 expression in astrocytes. The complex Cyclin D1/CDK4 induces cell cycle progression from G₁ phase to S phase through phosphorylation of Rb. As a consequence I conjectured that inhibition of ROCK would induce phosphorylation of Rb, overcoming CDK4 inhibition by P16. Furthermore, in

my experiments, P16 expression was decreased upon reprogramming. ROCK regulates cytoskeleton function by inhibition of myosin phosphatase. The activity of the latter de-phosphorylates the myosin light chain, an important component of the cytoskeleton. Inhibition of ROCK promotes myosin phosphatase activity leading to cytoskeleton remodelling (Wettschureck and Offermanns, 2002). These cytoskeleton changes promotes proliferation (Provenzano and Keely, 2011). Supranowicz et al. (2012) showed increased protein expression of many adult stem cell markers in primary human ectocervical cells reprogrammed with ROCK inhibitors in combination with feeder layers of irradiated fibroblasts. Some of these markers were integrins $\alpha 6$ and $\beta 1$, p63, CD44, β -catenin, Notch-1, delta like canonical Notch ligand 1 (DLL1). The authors also measured the expression of markers of pluripotent stem-cells (SRY box 2 [SOX2], Nanog homeobox [NANOG] and octamer-binding protein 4 [OCT4]) and did not observed up-regulation, suggesting that the reprogrammed cells were committed to an epithelial lineage. Although the issue of limited *in vitro* proliferation of HEECs was circumvented, reprogramming did not render the cells responsive to various differentiation stimuli.

No changes in proliferation and differentiation induction in HEECs with ovarian steroid hormone treatment were observed. An increased proliferation with E2, an inhibition of proliferation with P4 and even a further reduction in proliferation with the combination of both was expected, since progesterone has an anti-proliferative effect, and E2 induces synthesis of PR by action on an oestrogen response element in the promoter region (Kaster et al., 1990). Naive HEECs presented an arrested proliferation, therefore it would be hard to notice any inhibitory effect, considering the already low rate of growth of untreated cells. However, the unresponsiveness was also observed with reprogrammed HEECs. One possible reason for that, could be the observed variability in ovarian steroid receptors expression in HEECs. However, normal expressions of ER and PR were detected in cultured HEECs by ligand binding

assay (Zhang et al., 1995) and by immunocytochemistry (Hombach-Klonisch et al., 2005). Another explanation could be the need of the stromal compartment for the ovarian steroid hormones to enhance or reduce proliferation in the epithelial cells. It has been demonstrated in mice that oestrogens induce proliferation in the epithelial compartment of the endometrium through ER in the stromal cells (Cooke et al., 1997). In turn, P4 would inhibit epithelial growth by binding to its cognate receptor PRA in the endometrial stroma (Hantak et al., 2014).

Progesterone and hCG have been shown to induce glycodelin-A secretion in endometrial glands (Sepäälä et al., 2002). There is no evidence for glycodelin regulation by oestrogen (Seppälä et al., 2002). However, no up-regulation of *PAEP* with P4 or hCG was observed in my experiments. My results suggest that the decidualized endometrial stromal compartment also regulates epithelial differentiation. Which decidual soluble factors stimulate epithelial differentiation is still elusive. PRL was a rational candidate, but no response was detected with its use. Arnold et al. (2001) showed induction of glycodelin secretion by HEECs when co-cultured with undecidualized HESC, but the effect was only attained when the cells were seeded in Matrigel. This may indicate that HEECs need to be allowed to polarize, to respond to differentiation signals.

Conditioned medium from decidualized HESCs induced proliferation of HEECs. I speculate that this effect could be explained by PR isoform specific roles. While stromal PRA would only regulate epithelial proliferation by counteracting oestrogen-driven mitogenic effects (Vasquez and DeMayo, 2013), PRB would stimulate epithelial proliferation in an oestrogen-independent manner. Indeed, Mulac-Jericevic et al. (2000) demonstrated that specific ablation of PRA in mice led to progesterone-induced epithelial proliferation through PRB. Another plausible explanation for this effect and the partial reversal of senescence would be that the decidualized HESCs provided soluble growth factors for HEECs, in a similar way that feeder layers do for

embryonic stem cell cultures (Llames et al., 2015). As a matter of fact, decidualization triggers apoptosis in the stromal compartment as irradiation does in the fibroblasts of the feeder layer (Akcali et al., 2003). The main focus of the studies was on the mechanisms of apoptosis. The extrinsic pathway is involved, through translocation of TNF receptors and FAS receptors from the Golgi apparatus to the plasma membrane, and increasing levels of FASL and TNF during the mid-secretory phase (Boeddeker and Hess, 2015). The intrinsic pathway is also affected by decrease of anti-apoptotic B-cell lymphoma 2 (BCL2), increase of pro-apoptotic BCL2 associated X, apoptosis regulator (BAX) and release of cytochrome C from mitochondria (Tao et al., 1997). Despite the several studies of the decidual transcriptome and proteome (Popovici et al., 2000, Brar et al., 2001, Garido-Gómez et al., 2014, Gellersen and Brosens, 2014), the factors released by the apoptotic decidual cells that reverse epithelial senescence and induce proliferation of gland cells are yet undetermined.

Up-regulation of *PTGS2* was not observed, and induction of PGE₂ secretion was inconsistent in HEEC upon trypsin exposure. Ruan et al. (2012) showed a 3 fold induction in PGE₂ secretion and up-regulation of *PTGS2* in mouse endometrial epithelial cells treated with trypsin. Furthermore, the authors could inhibit these responses using amiloride, an ENaC inhibitor. In my study, ENaC expression was only observed in the borders of the HEEC monolayers, which could explain the blunted and inconsistent responses to the serine protease. ENaCs were identified in the apical membrane of HEECs (Enuka et al., 2012). Taken together these data suggest the need for epithelial polarity for these ion channels to function and be appropriately expressed in HEECs.

Mouse decidualization depends on embryo's presence (Lee et al. 2007), not occurring spontaneously. In humans, decidual differentiation occurs independently of pregnancy, in every ovulatory cycle, by the influence of P4 and cAMP (Gellersen et al., 2007). This physiologic difference may account for the unresponsiveness of

HEECs to trypsin. This role for serine proteases would not have been conserved, once the decidual control shifted from the embryo to the mother.

The luminal epithelium is the main uterine sensor for the embryonic signals, besides being the site for blastocyst attachment (Zhang et al., 2013). Structural differences between human luminal epithelium and endometrial glands are well described (Demir et al., 2002), and distinct transcriptome has been detected in mice (Niklaus and Pollard, 2006). In my study, the isolated HEECs derived from the dissociation of glandular clumps, conferring on them a specific phenotype. I conjecture that trypsin-triggered PGE₂ secretion could be a property of the luminal epithelium, which forms the first maternal embryo interface. Glandular cells would not retain this capacity.

Epithelial cells polarize to differentiate between the interior of the organism and the external environment. The apical membrane is exposed to the organ lumen and the basolateral membrane is in contact with the interior. A complex of adhesion molecules and organelles (adherent junctions, tight junctions, gap junctions and desmosomes) take part in this polarization process (Kaplan et al., 2009). G protein-coupled receptors, such as PARs and LHCGR, and ion channels, such as ENaCs, are bound to the apical membrane, while proteins involved in cell-to-cell communications reside in the basolateral membrane. The transport of molecules produced in the cells is also organized in a mode that some are secreted to the lumen and some are released to the underlying compartment, usually stroma (Nelson, 2009). This way, the epithelial cells can perform the roles by which they were differentiated for. Cells in monolayers develop a flat morphology that compromises polarity and, as a consequence, function. Several authors have reported dedifferentiation of endometrial cells in two dimensional (2D) cultures, limiting functional studies (Zhang et al., 1995, Classen-Linke et al., 1997, Arnold et al., 2001). That could be a reason for the dampened responses of the epithelial cells in monolayer cultures. On that account, the next logical step is the development of three dimensional (3D) culture systems for HEECs.

Chapter 4

Establishing Endometrial Gland Organoids

4.1 Introduction

In vivo, epithelial cells reside in a 3D environment that enables contact with extracellular matrix (ECM) and neighbouring cells. They are organized in a manner to allow for interaction with the exterior, through the lumen, and with the interior of the tissue through cross-talk with the underlying cells. This system requires cellular polarity. This is a complex process involving several players, such as partitioning defective proteins (Par proteins), Crumbs proteins (CRB), Scribble proteins (SCRIB), Rho GTPases, lipids, and signal pathways, e.g. Notch, Wnt and Hippo (Rodriguez-Boulán & Macara, 2014, Bayraktar et al., 2006). This arrangement facilitates efficient secretion of target molecules, relaying signals to the external environment through the apical membrane and to the underlying compartments across the baso-lateral membrane. At the same time, cues from the organ lumen are delivered to the cells onto the apical domain, and messages from neighbouring cells are sensed by the basolateral domain (Vreede et al., 2014).

In monolayer cultures, the cells are attached to the rigid flat surface of the dish, changing their shape and spreading. Interact with the ECM, and the contact with the adjacent cells are restricted (Pampaloni et al., 2007). These limitations are associated with loss of function and differentiation, altering the responses in different situations (Antoni *et al.*, 2015). In fact, cells can regain their differentiated phenotype *in vitro* when embedded in a 3D environment. This has been demonstrated with chondrocytes and mammary cells (Petersen et al., 1992, Caron et al., 2012, Sokol et al., 2016). In anticancer drug discovery, 95% of the compounds, first selected based on the effects observed in cell line 2D cultures, fail to be licensed due to poor responses in animal experiments or in clinical trials (Hutchinson and Kirk, 2011). The same problems associated with dedifferentiation have been described in endometrial epithelial cell cultures (Classen-Linke et al., 1997, Chitcholtan et al., 2012). For example, Arnold et al. (2001) demonstrated that HEECs could only respond to

differentiation signals from HESCs, when both cell types were cultured in Matrigel. This reconstituted basement membrane preparation is extracted from the Engelbreth-Holm-Swarm mouse sarcoma. It is compound of approximately 60% laminin, 30% collagen IV, and 8% entactin. It also contains heparan sulfate proteoglycan, TGF β , EGF, IGF1, FGF, nerve growth factor (NGF), PDGF, and tPA, in variable concentrations (Kleinman et al., 1986, Vukicevic et al., 1992).

3D endometrial gland models can help us to understand the roles of these structures in implantation and early pregnancy. Endocrine and paracrine interactions with other components of the reproductive system, namely the ovaries, the endometrial stromal compartment and the implanting embryo, could be assessed *in vitro*, in a rather more physiologic context than in 2D cultures.

The establishment of gland-like organoids has been described frequently in the last decade due to their pivotal role in cancer research. Prostate, intestine, colon, gastric, and mammary cell organoids are some of the recently described gland-like structures (Karthaus et al., 2014, Sato et al., 2011, Bartfeld et al., 2015, Sokol et al., 2016). These organoids were developed in well-defined culture conditions, combining basal membrane extract with medium containing factors that stimulate proliferation of progenitor cells (R-spondin 1, Noggin), de-differentiate transit amplifying cells into progenitor cells (EGF), and avoid cellular senescence (nicotinamide), along with molecules that induce organ-specific gland differentiation. FGF10 and HGF are expressed by the endometrial stroma, and are key factors in endometrial gland morphogenesis (Gray et al., 2001). One study described the establishment of fallopian tube organoids by modulation of Wnt pathway, and also suggested a role for Notch in cell fate determination (Kessler et al., 2015).

One of the main roles of endometrial glands is to provide histiotrophic support to the embryo, until the haemochorial placenta is established, around 12 weeks of pregnancy (Burton et al., 2002).

Missed miscarriage is characterized by foetal demise, without expelling of the products of conception. It is usually suspected when the symptoms and clinical signs of pregnancy are regressing, and the diagnosis is confirmed by an ultrasound scan (Griebel *et al.*, 2005). The exact incidence of chromosomal abnormalities in these cases is undetermined. Karyotype studies of the products of conception detected 60-75 % of chromosome errors in isolated cases of missed miscarriage (Phillipp and Kalousek, 2001, Ferro *et al.*, 2003, Phillipp *et al.*, 2003, Lathi *et al.*, 2007). Considering that only 2–4% of the couples suffering recurrent miscarriage are carriers of balanced translocations, the real burden of the chromosomal defects might be even smaller in recurrent missed miscarriage. So, 25 – 40 % of the missed miscarried embryos present normal karyotypes, indicating that other causes may be involved in the aetiology. I postulate that defects in endometrial glands impair histiotrophic support for the genetically normal embryo, eventually leading to demise.

This chapter explores the options for endometrial epithelium 3D culture, the establishment of a method for culture of endometrial gland-like organoids, and investigates the responses of these epithelial structures to differentiation signals. I also suggest that endometrial gland clonogenicity is disturbed in recurrent missed miscarriage cases.

4.2 Results

4.2.1 HEEC Spheroids

My first attempt to establish a 3D model for HEECs was through spheroid formation. Spheroids are aggregates of mutually adherent cells that do not attach to culture substrate, and adopt a spherical-like shape (Fennema et al., 2013). These cell clusters can mimic *in vivo* solid tumours, being a useful choice for studying anticancer therapy sensitivity and resistance (Adcock et al., 2015). The potential use in regenerative medicine has widened the interest in non-cancerous cell organoids. Recent studies with hepatocytes, thyrocytes and chondrocytes have been published (Bell et al., 2016, Wang et al., 2016, Bartz et al., 2016).

A specific plate designed for hanging drop culture was used (Figure 4.1A) and 10^4 primary isolated HEECs / well were seeded in 45 μ l of final CRC medium supplemented with 2.5 % Matrigel. The cells started to organize in sphere-like structures within 48 hours (Figure 4.1 C). Spheroids were transferred to 96-well plates containing Matrigel, after 4 days, for treatment. To test if organoid formation would render the HEECs responsive to differentiation cues I treated them with different combinations of E2, MPA and hCG for 24 hours. No induction of *PAEP* was observed (Figure 4.1B).

Next I examined the effect of the decidualized stromal compartment on spheroid differentiation. After being transferred to Matrigel, the spheroids were cultured for 24 hours in conditioned medium from HESC decidualized for 4 days. No significant induction of *PAEP* was detected (Figure 4.1D). The data indicate that, although organized in a 3D arrangement, the HEEC spheroid do not respond to various differentiation signals.

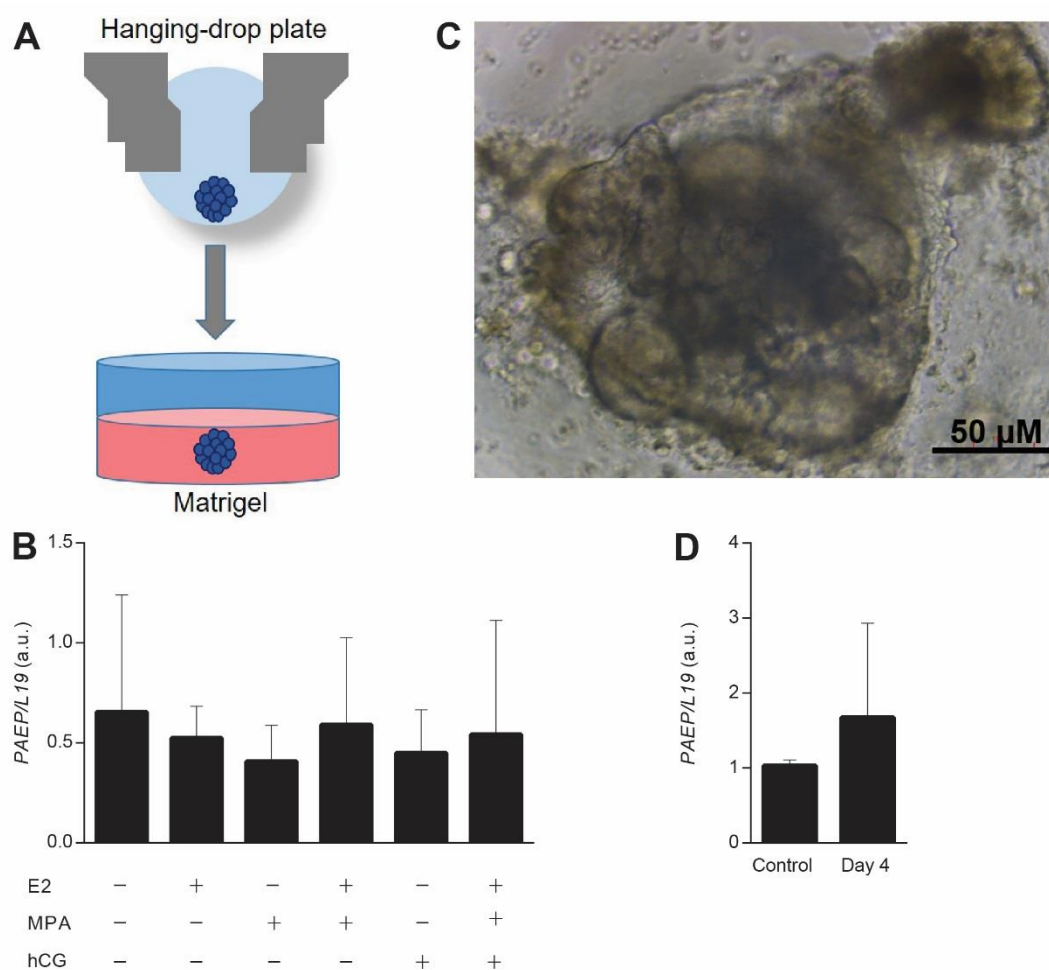


Figure 4.1 Unresponsiveness of HEEC spheroids to differentiation cues. (A) Cartoon showing the culture of HEEC spheroid in hanging-drop and its transfer to cell culture microplate containing Matrigel. **(B)** Bright field image of a compact HEEC spheroid in a hanging drop, 2 days after seeding of 10^4 cells / well of a hanging drop plates. **(C)** Normalized expression of *PAEP* in HEEC spheroid treated with different combinations of 10 nM E2, 1 μ M MPA and/or 1 IU / ml hCG for 24 hours ($n = 3$). Their presence or absence is shown by + or – in each column. $P = ns$ (Friedman test) **(D)** Normalized expression of *PAEP* in paired HEEC spheroids cultured for 24 hours with or without (control) conditioned medium from HESC decidualized for 4 days ($n = 4$). Data are presented as mean \pm SD. $P = ns$ (Wilcoxon signed-rank test).

4.2.2 Endometrial Gland Organoids Established by the Use of Matrigel and HGF Supplemented Medium

A variety of methods for creating human endometrial gland organoids have been reported (Rinehart et al., 1988, Classen-Linke et al., 1997, Bläuer et al., 2005, Valentijn et al., 2015), all of them based solely on the use of Matrigel. The efficiency rate of organoid forming is not described and the performance on functional assays was diverse.

Sugawara et al. (1997) developed human endometrial gland-like organoids using Matrigel and HGF from three different biopsies. Using a similar method, I attempted to establish gland organoids. Ten thousand primary isolated HEEC / well mixed in 50 μ l Matrigel were seeded in 96-well plates. The cells were overlaid by final CRC supplemented with 50 ng/ml HGF. HGF is an endometrial stroma derived factor and is involved in uterine gland morphogenesis in ovines (Taylor *et al.*, 2001). The cells initially clustered and, gradually, some of them showed central lumen formation. The organoids were mostly round, with a central lumen (Figure 4.2A). However, only 27.3% (3/11) of the cultures formed gland-like structures after 7 days.

To examine the response of these 3D cell formations to differentiation cues, the organoids were treated with different combinations of E2, MPA and hCG for 24 hours, and no induction of *PAEP* was verified (Figure 4.2B).

To investigate the effect of cues from the decidualized stroma, the organoids were cultured for 24 hours in conditioned medium from HESCs decidualized for 4 days. Once more, despite a trend of higher expression on treated organoids, no significant *PAEP* up-regulation was observed, suggesting that, in addition to the low efficacy for forming the gland organoids with this method, this 3D arrangement do not confer on HEECs the ability to respond to differentiation signals from diverse sources (Figure 4.2C).

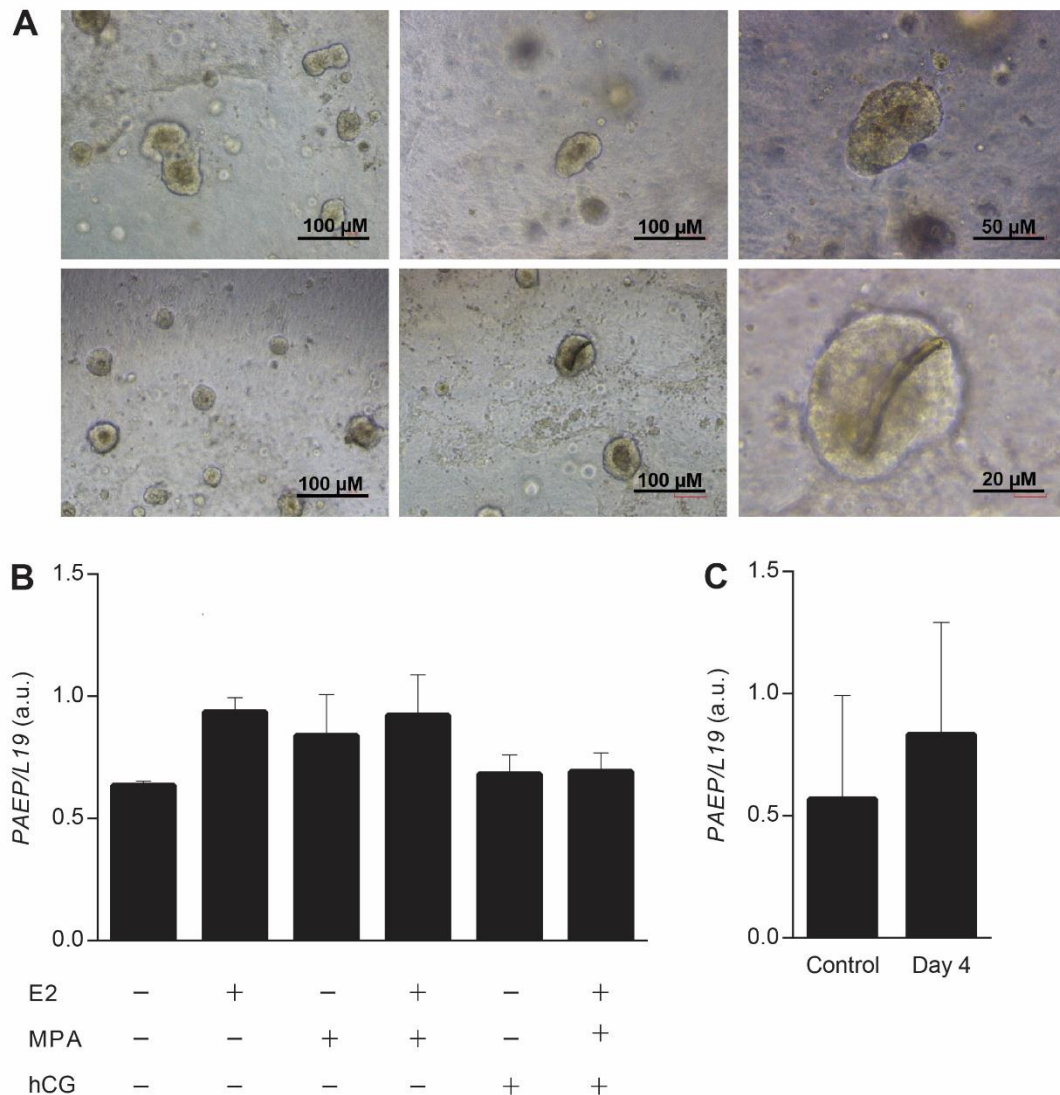


Figure 4.2 Unresponsiveness of HEEC organoids in Matrigel and HGF supplemented medium to differentiation cues. (A) Bright field images of endometrial gland organoids in Matrigel for 5 days. Ten thousand cells / well were seeded into a 96-well plate with 50 μ l Matrigel / well, and the culture medium was supplemented with 50 ng / ml HGF (representative of $n = 3$). **(B)** Normalized expression of *PAEP* in endometrial gland organoids treated with different combinations of 10 nM E2, 1 μ M MPA and / or 1 IU / ml hCG for 24 hours ($n = 3$). Their presence or absence in the medium is shown by + or – in each column. $P = ns$ (Friedman test) **(C)** Normalized expression of *PAEP* in endometrial gland organoids cultured for 24 hours with or without (control) conditioned medium from HESC decidualized for 4 days ($n = 3$). Data are presented as mean. \pm SD. $P = ns$ (Wilcoxon signed-rank test).

4.2.3 Cell Scaffolds for HEEC 3D Cultures

In vivo, the epithelial cells lie in an intricate 3D fibrous meshwork of collagen and elastic fibres, the ECM. The combination of chemical composition and nanostructure supports biochemical and biophysical roles, such as transport of soluble molecules and absorption of mechanical stress (Lee et al., 2007). Scaffolds have been developed to emulate ECM functions, and generate 3D cell cultures. Intense research has been undertaken to improve the bulk material, pore size, nanoscale design and surface properties. One study described the development of a bovine endometrial construct, using a polyglycolide electrospun scaffold (Mackintosh et al., 2015). The scaffolds were populated with stromal and epithelial cells from bovine endometrium, and cells expressed vimentin and cytokeratin, besides accumulating prostaglandins in response to arachidonic acid, oxytocin and lipopolysaccharide.

I used scaffolds developed at the University of Warwick Engineering School, by Prof Neil Cameron and Dr Ahmed Eissa. Well-defined macro-porous foam scaffold discs were produced by emulsion templating technique, whereby a highly porous polymer is created from a monomer (triacylate), using water droplets (Figure 4.3A). The inability to monitor the cells growing in the scaffolds is a disadvantage of the method, so HEECs were initially seeded onto fibronectin coated scaffolds, cultured for 15 days in final CRC medium, and stained for haematoxylin/eosin (H&E) and CK18. The cells populated the discs, exhibiting a multilayer structure (Figure 4.3B). Another reported issue is the chance of the cells to flatten and spread in the scaffolds, behaving as though in 2D monolayer cultures (Stevens *et al.*, 2005). To address this concern, I cultured paired HEECs in monolayers and in scaffolds, immunostained them for CK18, and acquired Z-stack images of single cells by confocal microscopy. The cells in monolayers had an overt flat shape, in contrast with the cell in scaffolds, which displayed a distinct 3D configuration (Figure 4.3C).

Once the 3D structure of the HEECs in the scaffolds was demonstrated, functional assays were performed. First, I tested the differentiation of HEECs in scaffolds compared to 2D cultures. Paired HEECs were seeded in flat dishes and scaffolds, and *PAEP* expression was measured after 3 and 7 days in culture. A trend of higher expression of *PAEP* in scaffold-grown HEECs was observed, but the differences were not statistically significant (Figure 4.4A). Next, the responsiveness of HEECs in scaffolds to E2 and P4 was tested, and the impact of the stromal compartment on these responses was also addressed. Three groups of paired HEECs were seeded in scaffolds and grown for 22 days. One group was mono-cultured, and was not treated. A second mono-cultured group was treated with E2 for 15 days, followed by the addition of P4, for 7 days. The third group was co-cultured with HESC and was also treated with E2 and P4. No difference in *PAEP* expression was detected upon E2 and P4 treatment, or upon co-culture with HESC (Figure 4.4B). To further explore the responses of scaffold-grown HEECs to differentiation cues, cells were grown on scaffolds for 15 days, then treated for 24 hours with conditioned media of HESC decidualized for 4 days. Although a trend of higher expression of *PAEP* in treated HEECs was again observed, statistical significance was not reached (Figure 4.4D). Inferring that the HEECs in scaffolds were polarized, and considering that polarization is pivotal for expression of apical domain proteins such as ENaCs, I investigated the effect of trypsin in this 3D model. HEECs were grown in scaffolds for 15 days, and were treated with trypsin. PGE₂ concentration in the culture supernatant collected 24 hours after the treatment was measured by ELISA. No enhancement in the prostaglandin secretion was detected (Figure 4.4C).

The data indicate that, although HEECs grow in a 3D conformation in scaffolds, they remain unresponsive to differentiation signals using this model.

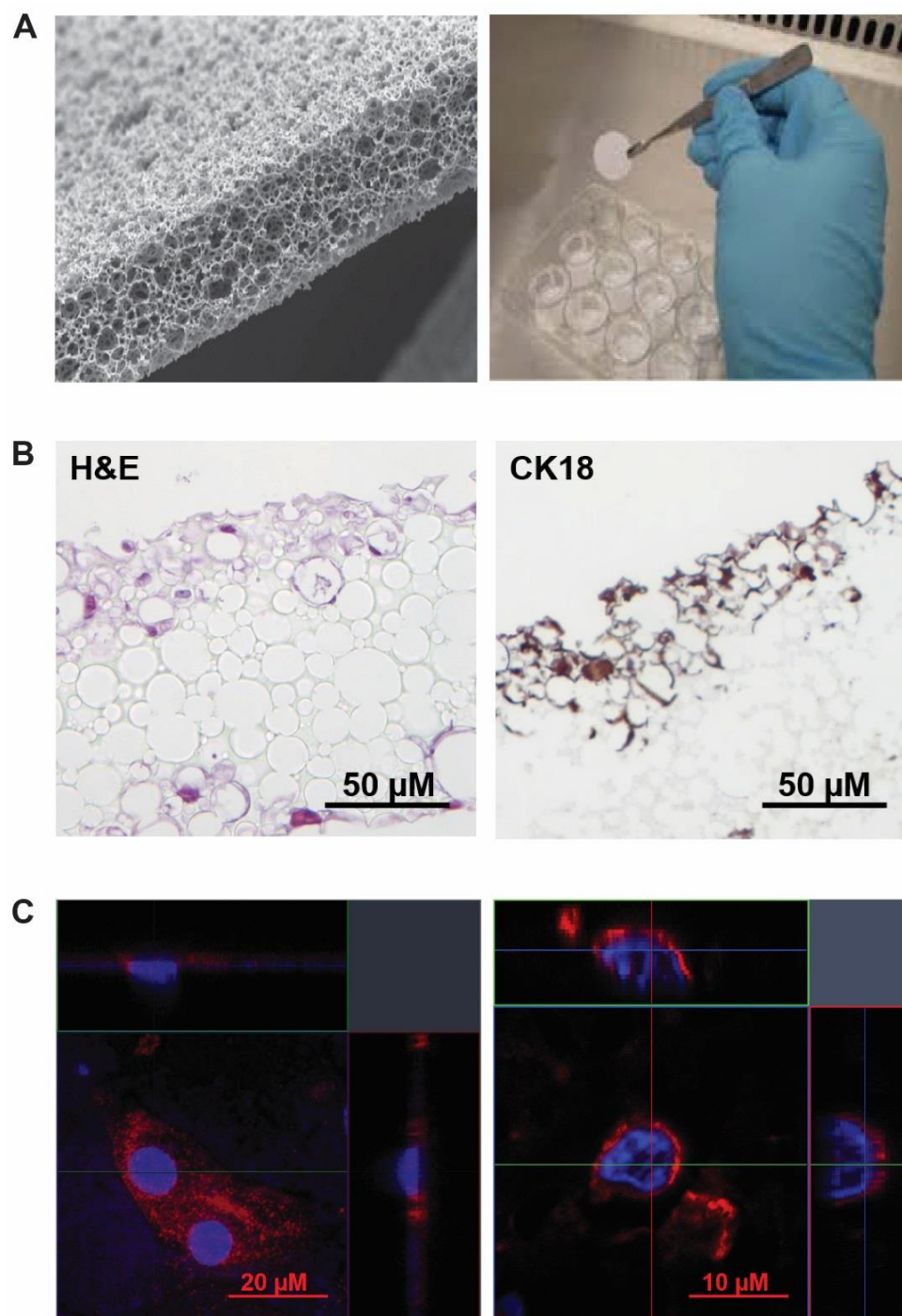


Figure 4.3 HEEC growth and population of thiol-acrylate cell scaffolds. (A) Pictures showing the microporous structure of the scaffold and the scaffold discs ready to be used. Discs were cut to 1 cm diameter, 200 μm thick and were placed inside 24-well plates for cell culture. **(B)** HEECs cultured in a scaffold for 15 days and stained with H&E (left) and CK18 (right panel). **(C)** Immunofluorescence of CK18 in HEECs cultured as monolayer, showing flat architecture (left panel), and in the scaffold exhibiting 3D conformation (right).

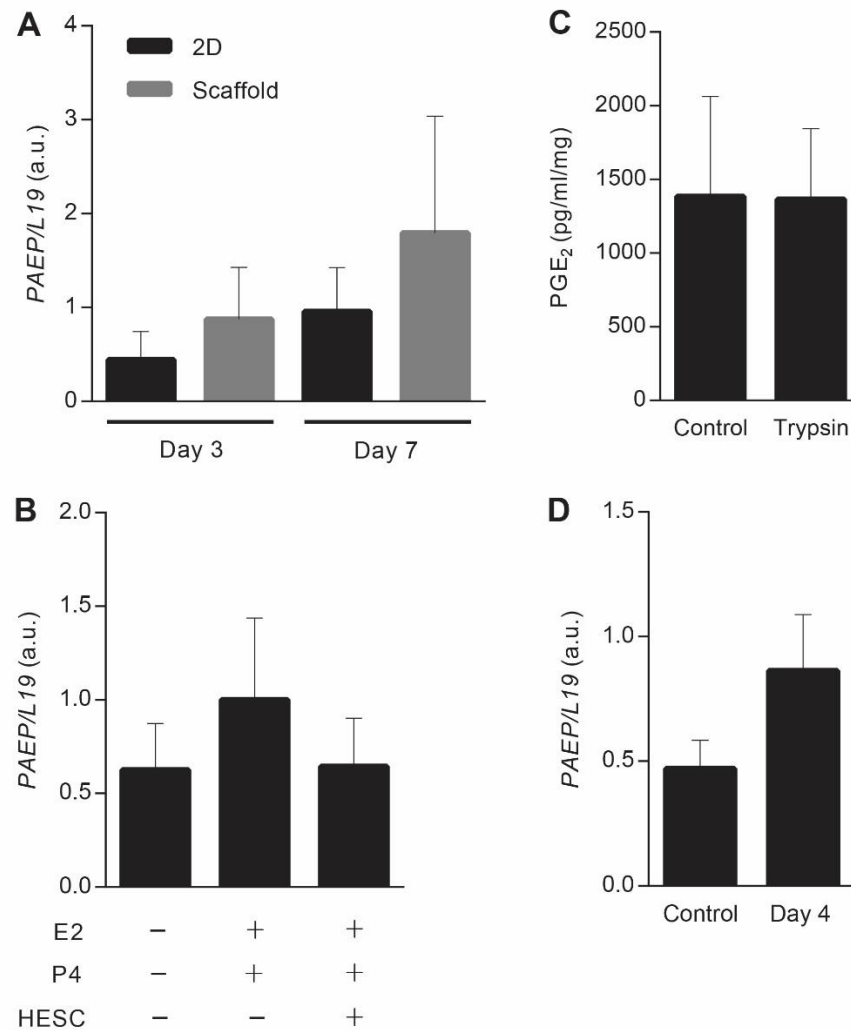


Figure 4.4 Responses of HEECs in scaffold to differentiation cues, and comparison with 2D culture. (A) Normalized expression of *PAEP* in HEECs in monolayers and in scaffolds. Paired HEECs were cultured in scaffolds and monolayers ($n = 3$). mRNA was harvested after 3 and 7 days. **(B)** Normalized expression of *PAEP* in paired HEECs cultured in scaffolds and either untreated or treated with 10 nM E2 for 14 days, followed by 10 nM E2 and 1 μ M P4 for 7 days. One of the treated groups was co-cultured with HESCs ($n = 3$). **(C)** Secretion of PGE₂ by HEECs cultured in scaffolds and either untreated (control) or treated with 10 nM trypsin in single pulse lasting 10 minutes ($n = 3$). Culture supernatant was collected 24 hours after the treatment. Data are presented as mean secretion normalized to total protein in cell lysates \pm SD ($n = 3$). **(D)** Normalized expression of *PAEP* in HEECs cultured in scaffolds and either untreated (control) or treated for 24 hours with conditioned medium from HESCs decidualized for 4 days ($n = 3$). Data are presented as mean \pm SD. $P = ns$.

4.2.4 Establishment of Endometrial Gland Organoids Using Matrigel and Modified Adult Stem-Cell Medium

Since Matrigel and HGF were not sufficient to derive responsive endometrial gland-like organoids efficiently, I decided to try another method to generate these gland-like structures. The use of factors that stimulate cell stemness has been recently reported for gastric-, intestine-, colon-, mammary gland- and prostate-like organoids (Karthaus et al., 2014, Sato et al., 2011; Bartfeld et al., 2015, Sokol et al., 2016). Specific factors to induce organ-specific gland differentiation were also deployed. HGF and FGF10 have been demonstrated to be key factors in endometrial gland morphogenesis.

Using Matrigel and culture medium supplemented with R-spondin 1, Noggin, EGF, nicotinamide, a TGF β inhibitor (A83-01), HGF and FGF10, henceforth referred as expansion medium, it was possible to derive endometrial gland-like organoids from primary HEECs, with a 97.4% (38/39) efficiency. The organoids grew in culture, progressively increasing their number and size. Round shape was more often observed, but elongated organoids were also detected, depicting a feature that recapitulates *in vivo* endometrial glands (Figure 4.5A). The organoids were immunostained, and showed expression of CK18 and E-cadherin, a key component of adherens junctions (Figure 4.5B).

To test the responsiveness to differentiation cues, organoids in expansion medium were treated with E2, P4, cAMP, PRL or hCG for 8 days. A two-fold increase in *PAEP* expression was observed with cAMP (Figure 4.6A). To further address the issue, I treated organoids with different combinations of E2, P4, cAMP, PRL hCG, and observed that E2, P4 and cAMP in combination induced a 3 fold increase in *PAEP* expression. The addition of PRL or hCG did not further enhance the *PAEP* induction (Figure 4.6B). To explore the effect of differentiation signals from the endometrial stroma, organoids were cultured for 24 hours in conditioned medium from HESC

decidualized for 4 days. A trend of higher *PAEP* expression in treated cells were observed, but the difference was not statistically significant (Figure 4.6 C).

Glycogen is a key component of endometrial gland histiotrophe, produced in high quantities during the secretory phase (Burton et al., 2011). Endometrial gland-like organoids were grown for 7 days, treated with E2, P4, cAMP, PRL and hCG for 8 days. The organoids were lysed and cell glycogen levels were measured by a colorimetric assay. No differences were observed in organoid glycogen concentrations upon stimulation with differentiation signals (Figure 4.6 D).

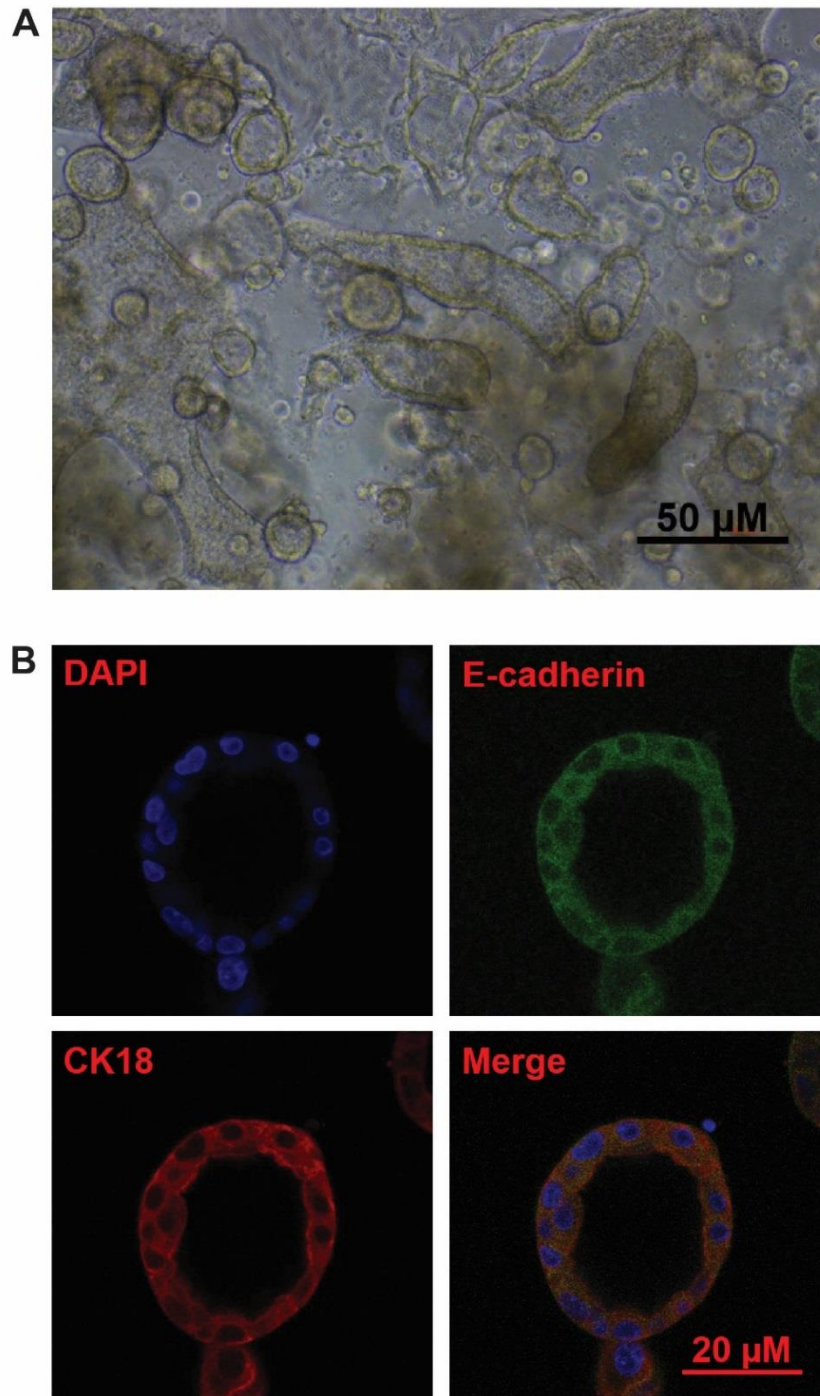


Figure 4.5 Establishment of endometrial gland organoids using Matrigel in association with a modified adult stem cell medium. (A) Bright field image of endometrial gland organoids cultured in Matrigel and supplemented with expansion medium for 7 days. **(B)** Immunofluorescence of CK18 and e-cadherin in endometrial gland organoids, counterstained with DAPI. Organoids were cultured Matrigel for 7 days, fixed and stained as a whole mount and the images were acquired with a confocal microscope.

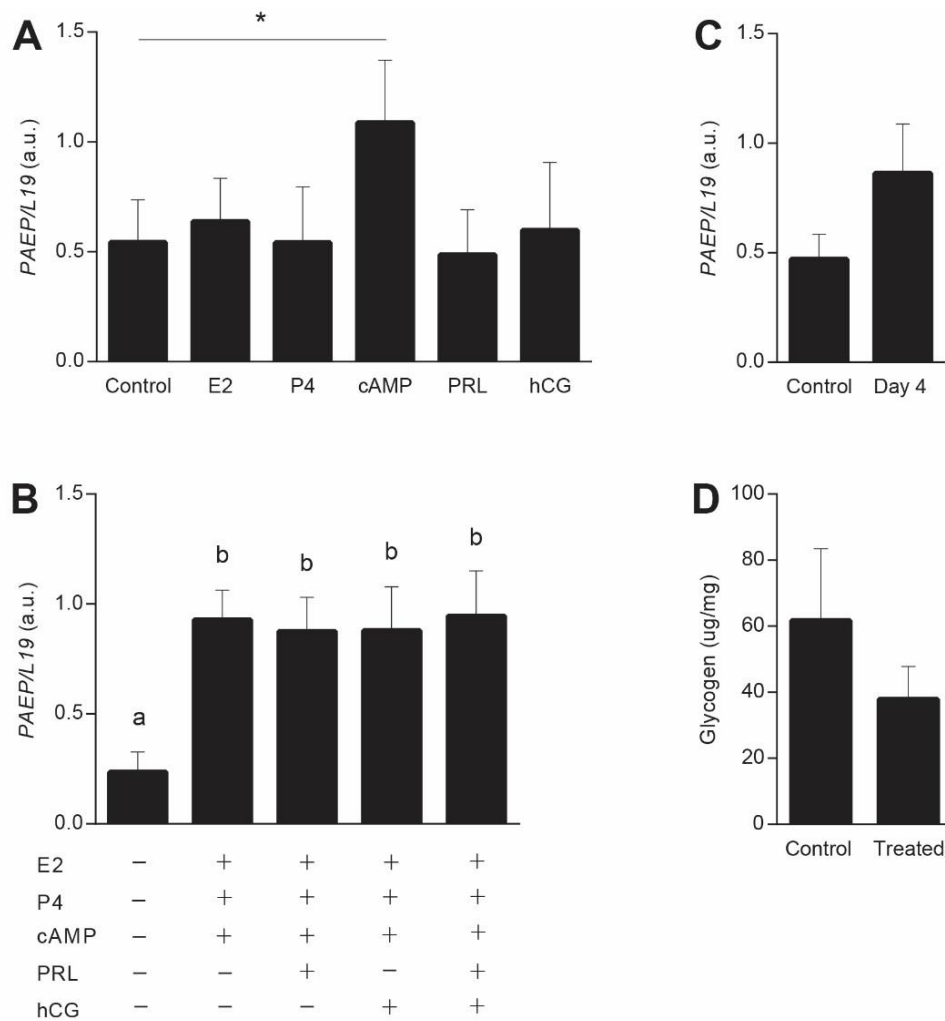


Figure 4.6 Responses of endometrial gland organoids in Matrigel and expansion medium to differentiation cues. (A) Normalized expression of *PAEP* in paired organoids either untreated (control) or treated for 8 days with 10 nM E2, 1 μ M P4, 0.5 mM cAMP, 20 ng / ml PRL or 1 IU / ml hCG ($n = 3$). Data are presented as mean. \pm SD. $*P = 0.047$. **(B)** Normalized expression of *PAEP* in organoids treated for 8 days with different combinations of 10 nM E2, 1 μ M P4, 0.5 mM cAMP, 20 ng / ml PRL and 1 IU / ml hCG ($n = 3$). Their presence or absence is shown by + or – in each column. Different letters above error bars indicate that those groups are significantly different from each other. $P = 0.3$. **(C)** Normalized expression of *PAEP* in organoids cultured for 7 days, followed by treatment for 24 hours with or without (Control) conditioned medium from HESC decidualized for 4 days ($n = 3$). Data are presented as mean \pm SD. $P = \text{ns}$. **(D)** Glycogen levels in organoids either untreated (Control) or treated with 10 nM E2, 1 μ M P4, 0.5 mM cAMP, 20 ng/ml PRL and 1 IU/ml hCG for 8 days. Data are presented as mean glycogen concentration in organoids, normalized to total protein in organoid lysates \pm SD ($n = 5$). $P = \text{ns}$.

4.2.5 Reduction of Gland Clonogenicity in Recurrent Missed Miscarriage (RMM) Cases

The histiotrophic support of the embryo during the first trimester of pregnancy is provided by the endometrial glands (Burton et al., 2002). In missed miscarriage cases, embryonic or foetal demise occurs with no associated uterine bleeding (Griebel et al., 2005). Around 35% of these cases are not explained by chromosomal anomalies (Lathi et al., 2007). Based on those observations, I hypothesized that endometrial gland defects could account for a proportion of recurrent missed miscarriage cases (RMM), defined as 3 or more episodes of missed miscarriage.

I set up organoid forming efficiency assays (OFE) for patients with and without recurrent missed miscarriage (Table 4.1). One thousand cells mixed in 5 μ l Matrigel drops were plated in 96-well plates. The drops were left to set for 1 hour, and were overlaid with expansion medium supplemented with ROCK inhibitor to avoid anoikis (Koyanagi et al., 2008). OFE was calculated based on the percentage of formed organoids, after 10 days, in relation to the number of seeded cells (Figure 4.7A). The organoid forming efficiency was significantly decreased in the missed miscarriage sample derived organoids (Figure 4.7B). After the counting, the organoids in the OFE assay were either untreated or treated with differentiation medium (10 nM E2, 1 μ M P4, 0.5 mM cAMP, 20 ng / ml PRL and 1 UI / ml hCG) for 8 days. mRNA was harvested, extracted, assessed by spectrophotometry, and was deemed eligible for PCR if ratio 260/280 \geq 1.8. No difference in *PAEP* induction was observed between control individuals and missed miscarriage derived organoids. However, the induction of *PAEP* upon differentiation cues was markedly higher in both groups than that observed in the previous similar experiment (Figure 2.6B), reaching up to 58 fold change (Figure 4.7C).

This confirms that the endometrial gland-like organoids established with expansion media are responsive to differentiation cues. The data also suggest that the

population of endometrial gland cells from recurrent missed miscarriage patients may have progenitor cell deficiency, being incapable to meet the demand for histiotrophic support of the first trimester pregnancy.

Table 4.1. Clinical characteristics of patients from OFE assay.

Groups	Control (n=9)	RMM (n=9)	<i>P</i>
Age	34.5 (32-43)	32 (28-45)	<i>P</i> = 0.7425
BMI	20 (18-29)	25 (17-34)	<i>P</i> = 0.2338
Day of cycle	LH+9 (7-10)	LH+9 (7-11)	<i>P</i> = 0.9536
Miscarriage	1 (0-1)	6 (4-8)	<i>P</i> < 0.0001
Live birth	0 (0-1)	0 (0-1)	<i>P</i> > 0.9999

Data are presented as median (range). BMI: body mass index

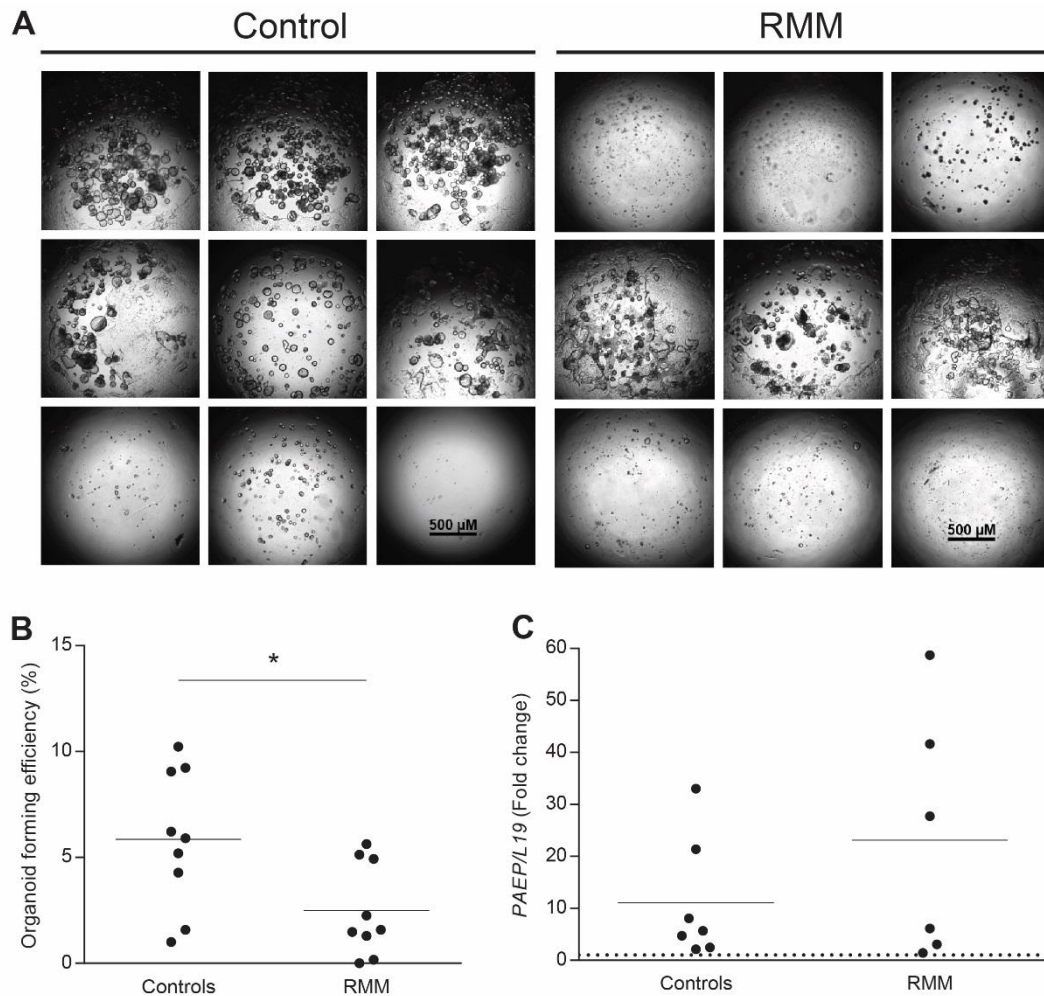


Figure 4.7 Endometrial gland cell clonogenicity in missed miscarriage and induction of differentiation in gland-like organoids. **(A)** Bright field images of endometrial gland organoids seeded in low density (1,000 live cells in 5 μ l Matrigel droplets) for organoid forming efficiency (OFE) assay, after 10 days. Left panel: control subjects; right panel: recurrent missed miscarriage cases **(B)** Organoid forming efficiency in control subjects and RMM cases ($n = 9$ in each group). $*P = 0.029$ (two sample t student test). **(C)** Fold induction of *PAEP* in endometrial gland organoids in control subjects ($n = 7$) and RMM cases ($n = 6$). The organoids were grown in expansion medium for 10 days, and were either untreated or treated with differentiation medium for 8 days. Data are presented as fold change compared to untreated samples (dotted line). $P = \text{ns}$ (two sample t student test).

4.3 Discussion

In this chapter, I reported the strategies to establish a 3D model that recapitulated the endometrial gland phenotype *in vitro*. Polarity is a pivotal property for gland cells to perform their roles efficiently. Although 3D cultures provide a more propitious environment for cells to emulate *in vivo* organization, every function may not be restored. The creation of a perfect artificial cell niche in culture dishes is a particularly challenging goal. Some variants can be partially controlled, but indeed, it is most likely that several required conditions are not uncovered yet.

HEECs formed compact spheroids in hanging-drops that could be transferred to Matrigel for treatments, however these aggregates did not show expected responses to differentiation cues. One possible reason could be the inability of the molecules to diffuse, and reach the cells residing in the core of the spheroids. The lack of vascularity makes diffusion the only available mean to support oxygen and nutrient delivery, and CO₂ and waste removal. Cells in different depths are in dissimilar nutritional state and, therefore, not at same stages of cell cycle (Edmondson et al., 2014). This is actually beneficial when testing a new anticancer drug, since it mimics a solid tumour environment (Mehta et al., 2012). But for testing the global effect of an agent on a normally polarized cell type, it may represent a confusion factor. The cells in the centre of the spheroid are not exposed to the treatment, and could mask the effect obtained on their surface counterparts.

The initial method for organoid deriving, using HGF only, proved to be highly ineffective. The only three endometrial samples that formed organoids were used for the assays, with no compelling results, so the method was set aside.

Most of the knowledge on matrix culture derives from studies with cancer cell lines. These cells show a rapid growth in culture, being the ideal choice for scaffold cultures. After the optimization for some culture parameters (number of seeded cells, medium

volume for seeding, time to add medium after the initial seeding, scaffold covering), fixing, and staining, it was possible to grow primary HEECs in the polymer scaffolds. The cells populated the scaffolds and exhibited a 3D conformation. Nevertheless, no response to differentiation cues was observed either. One reasonable cause for this refractoriness would be the inability of the tested molecules to diffuse through the scaffold and reach the deeper cells, as previously postulated for the spheroids. Microfluidic devices have been proposed as effective systems for uniform distribution of the cells inside the scaffold and for controlled delivery of biochemical signals (Tehranirokh et al., 2013). Another possible explanation would be a deficiency in cell-to-cell communication due to sub-optimal pore size. If the pores are too large, cells, even though in a 3D conformation, could not adhere to each other. Furthermore, another possible reason for the unresponsiveness of HEECs could be the inability to recapitulate the *in vivo* gland structure inside the macro-porous matrix. In this sense, the scaffolds would be more appropriate for luminal epithelium models.

Inhibition of TGF β signalling enhances stem cell self-renewal in humans (Sakaki-Yumoto et al., 2013). The canonical Wnt pathway, through the interaction of β -catenin with the transcript complex T-cell factor/lymphoid-enhancing factor (TCF/LEF), induces transcription of target genes involved in cell stemness (Van Camp et al., 2014). Noggin inactivates bone morphogen protein 4 (BMP4), whereas A83-01 is an inhibitor of TGF β receptors type I. R-spondin 1 is a Wnt/ β -catenin agonist, binding to leucine-rich repeat-containing G-protein coupled receptors (LGR). EGF was demonstrated to convert transit amplifying cells into multipotent stem cells (Doetsch et al., 2002). Nicotinamide prevents cell senescence by repressing P53, P21 and P16 and accelerating proliferation of pluripotent cells (Son et al., 2013). HGF and FGF10 are pivotal factor in endometrial gland morphogenesis (Taylor et al., 2001). Using a basement membrane extract (Matrigel), modulating the paracrine TGF β and

Wnt pathways involved in the stem cell niche, and providing cell fate cues, polarized endometrial gland-like organoids were efficiently established from primary HEECs.

The organoids initially showed modest gland differentiation responses that seemed to be exclusive to cAMP. Nevertheless, when the organoids in the forming efficiency assay were treated with a combination of differentiation cues, the response was considerably increased, with *PAEP* expression fold change of up to 58. This might be explained by the pronounced heterogeneity of the responses from sample to sample. Even in the OFE assays, some of the organoids produced only a moderate induction of *PAEP*. Attached HEECs, forming a monolayer underneath and at the border of the Matrigel drops, was often observed. This phenomenon was not observed in the OFE assays, most likely because the number of seeded cells was smaller, and therefore insufficient to establish a monolayer. This difference could be therefore accounted for the mild effect of differentiation signals on the organoids. It is possible that the 2D cells harvested along with the organoids masked the real response of the gland-like structures.

Glycogen is broken down within the gland cells by glycogen phosphorylase, soon after synthesis, and it is further degraded into diffusible sugars by α -amylase, at the apical surface (Jones et al., 2015). Gland activity is additionally enhanced by placental signals, in order to make this sugars available for the embryo (Jones et al., 2010). Glycogen accumulation is actually identified in the placenta as well as the endometrial glands during the first trimester of pregnancy (Burton et al., 2011). These observations could justify the fact that the glycogen levels were not higher in differentiated organoids.

Very little is known about missed miscarriage etiopathogenesis, and even less about recurrent cases. In this study, I detected differences in the OFE between recurrent missed miscarriage samples and control subjects. Based on this observation I postulate that endometrial glands of patients suffering recurrent missed miscarriage

have a deficiency of progenitor cells, compromising the ability to produce new gland cells through asymmetric division and thereby meeting the increased demand for uterine secretions in early pregnancy.

Chapter 5

Transcriptome of Human Endometrial Glands in the Secretory Phase and in Recurrent Missed Miscarriage

5.1 Introduction

Once I established a method for studying the endometrial glands *in vitro*, and demonstrated differences in gland cell clonogenicity between control subjects and women who suffered RMM, I decided to investigate these endometrial structures *in vivo*.

During the implantation process the endometrium must shift to a receptive state in order to accommodate the blastocyst. The uterine luminal epithelium is the first cell layer that comes in direct contact with the blastocyst. It is considered a gatekeeper, only allowing the endometrium to be breached during the WOI. Modifications are observed in this specialized epithelium, in order to prepare for adhesion and invasion (Zhang et al., 2013a). At the same time, changes arise in the underlying stroma, to make it ready for the embryo arrival. Angiogenesis and increased vascular permeability, along with uNK cell influx and mesenchymal-to-epithelial transition of fibroblasts, comprise the main transformations identified in the decidualized stroma, in response to the rising levels of P4, and local production of cAMP (Aplin, 2010).

A role for endometrial glands in the establishment of the WOI has been demonstrated in studies with mice and sheep. Animals were exposed to P4 or progestins in the neonatal period, which inhibited uterine epithelium proliferation, and as a consequence, adenogenesis. The animals were mated, and a common observation during pregnancies was that fertilization occurred, but the conceptuses were unable to implant (Filant and Spencer, 2014). P4 up-regulates expression of many genes in the glands, and some of the translated proteins could induce a receptive phenotype in the luminal epithelium. Which gland-derived factors are involved is still not fully elucidated, but LIF has been suggested, since *Lif* null mice present blastocyst implantation problems. In humans, uterine luminal fluid protein content changes along the menstrual cycle, and is altered in infertile women (Hannan et al., 2011).

Endometrial glands provide the histiotrophic support for the foetus during the first trimester of pregnancy (Burton et al., 2002). Defective endometrial gland function, described as luteal phase defect, is considered an underlying cause of early pregnancy loss (Burton et al., 2007). Incidence of chromosome abnormalities in sporadic miscarriages is around 50 %. No increase is observed in cases of recurrent pregnancy loss (Stephenson et al., 2002). The exact incidence of these genetic errors in cases of recurrent missed miscarriage is undisclosed, although 60 % – 75 % of chromosome errors were detected in isolated cases of missed miscarriage (Phillipp and Kalousek, 2001, Ferro et al., 2003, Phillipp et al., 2003, Lathi et al., 2007). This means that a significant proportion of missed miscarriage cases (25-40 %) might present with another cause. Impaired secretion of angiogenic factors and shifting from a Th2 to a Th1 immune response during the pregnancy were suggested (Paradisi et al., 2003, Fang et al., 2013, Daponte et al., 2013, Zhu et al., 2014). However, all the studies measured plasma concentrations of the mediators after the diagnosis of missed miscarriage, aiming to find a biomarker for the condition. Therefore, it is not possible to assert if they were a cause or a consequence.

Laser microdissection (LMD) is a technique that allows for the isolation of one cell type or microstructure within a complex tissue. The acquisition of pure samples is pivotal for characterization of expression profiles. Coupled with high throughput technology, LMD is a powerful tool for genomic, transcriptomic and even proteomic research (Curran et al., 2012). This procedure has been applied in the investigation of the endometrium physiology. Niklaus and Pollard (2006) used the method to uncover distinct molecular signatures for the luminal and glandular epithelium in mice. Yanaihara et al. (2004) compared the epithelial with the stromal compartment in human endometrium, using microarray gene analysis. Franchi et al. (2008) characterized the expression changes of specific genes (*DAF*, *SPP1*, *IL15* and *PGR*) in endometrial glands and stroma, during the window of implantation, using RT-qPCR.

In this chapter, I compare the transcriptome of LMD captured human endometrial glands in the early, mid- and late secretory phase. A gene signature of these glands in the receptive phase was revealed, reinforcing the importance of this cellular compartment in the establishment of the WOI. Next, I explore the differences in gene expression between mid-secretory endometrial glands from control subjects and women who suffered RMM. A strong mitochondria-related gene signature suggests that altered glandular bioenergetics is involved in the genesis of this form of miscarriage.

5.2 Results

5.2.1 Temporal Changes in Endometrial Gland Transcriptome, During the Secretory Phase of the Menstrual Cycle

Genomic modifications of the endometrium along the secretory phase have been extensively demonstrated (Riesewijk et al., 2003, Mirkin et al., 2005, Talbi et al., 2006, Haouzi et al., 2009, Díaz-Gimeno et al., 2011, Hu et al., 2014). It is logical to infer that the glandular compartment is responsible for several of these changes, since numerous of the DEGs are strongly expressed by endometrial glands. To confirm this, I used LMD coupled with RNA-sequencing to study the transcriptome of glands in the early, mid- and late secretory phase.

Three LH surge-timed human endometrial biopsies were acquired from each time point: LH+5, LH+8 and LH+11, corresponding to early, mid- and late secretory phase respectively. Table 5.1 displays the clinical characteristics of the patients. The samples were flash-frozen in liquid nitrogen and were kept in - 80 °C until LMD. Frozen sections were obtained from the samples, and were mounted on membrane slides. Endometrial glands were captured by laser microdissection (Figure 5.1). mRNA was extracted, and integrity was assessed at the Genomics Research Facility, Warwick School of Life Sciences, using Agilent 2100 Bioanalyzer with a RNA 6000 Nano chip. The samples were deemed suitable for sequencing if RNA integrity number (RIN) > 5.0 and 28S/18S rRNA ratio > 0.9 (Supplementary table 5.1). Complementary DNA synthesis and amplification were also performed by Genomics Research Facility, Warwick School of Life Sciences, using Ovation RNA-Seq System V2 (Nugen Technologies). Library preparation and sample barcoding were produced using the Ovation Ultralow System V2 1-16 (Nugen Technologies). The libraries were sent to the Wellcome Trust Centre for Human Genomics – High Throughput Genomics, Oxford, UK for 100pb paired sequencing on Illumina HiSeq 4000 system. Data were processed and analysed in collaboration with Dr Pavle Vrljicak.

Principal component analysis showed the libraries clustering separately according to the time-point of the secretory phase (Figure 5.2). A total of 702 DEGs in at least one pair-wise comparison were detected, using the online tool DESeq2 and Bonferoni correction. Between LH+5 and LH+8, 565 genes were differentially expressed. Between LH+5 and LH+11, there were 295 DEGs, and between LH+8 and LH+11, 70 DEGs were identified.

Using K-means cluster analysis, 5 different clusters were found representing the temporal expression pattern of the DEGs (Figure 5.3). The different patterns suggest a more pronounced change from early to mid-secretory phase than from mid- to late secretory phase.

Table 5.1 Clinical characteristics of patients from LH-timed endometrial gland assay.

Groups	LH+5 (n=3)	LH+8 (n=3)	LH+11 (n=3)	Statistical significance
Age	36 (32-40)	38 (34-43)	33 (32-38)	No
BMI	26 (25-29)	24 (23-30)	23 (22-25)	No
Miscarriage	5 (1-15)	0 (0-1)	4 (2-5)	No
Live birth	0	0	0 (0-1)	No

Data are presented as median (range)

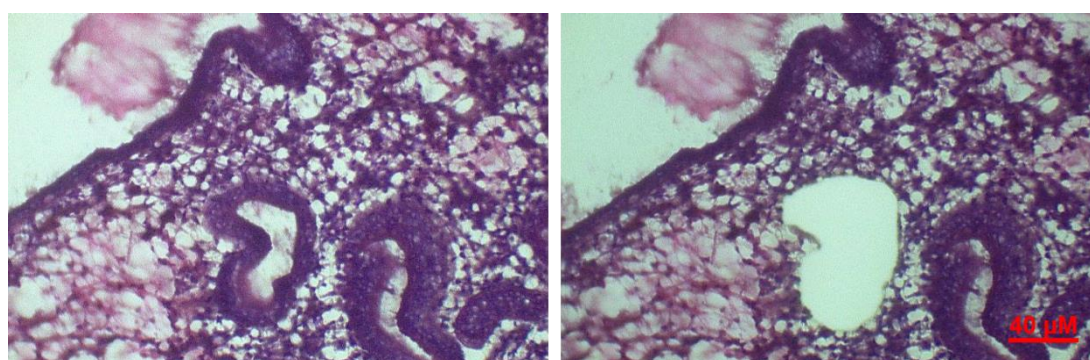


Figure 5.1 LMD of human endometrial glands. (A) Bright field image of a 10 µm section of human endometrium stained with cresyl violet and eosin-Y. **(B)** The same endometrial section from the previous image with a laser microdissected gland.

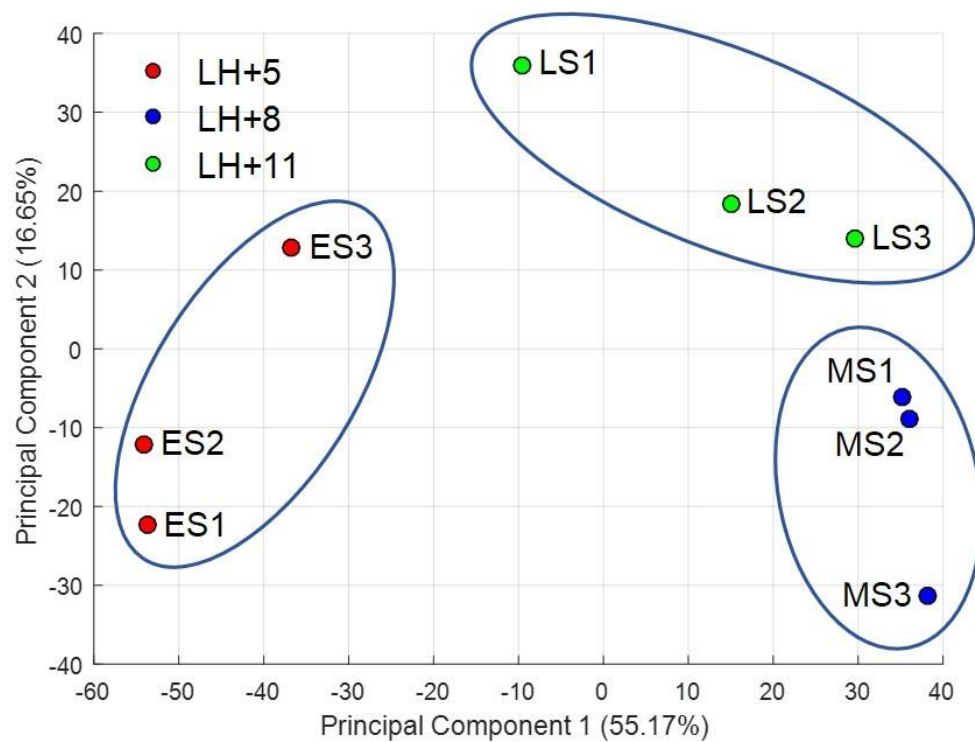


Figure 5.2 Principal component analysis of RNA-seq data from LH-timed laser microdissected endometrial glands in the secretory phase of the menstrual cycle. Principal component analysis of RNA sequencing data from human endometrial glands captured in the secretory phase of the menstrual cycle segregates the libraries according to the time-points in the secretory phase (LH+5, LH+8 or LH+11). ($n = 9$).

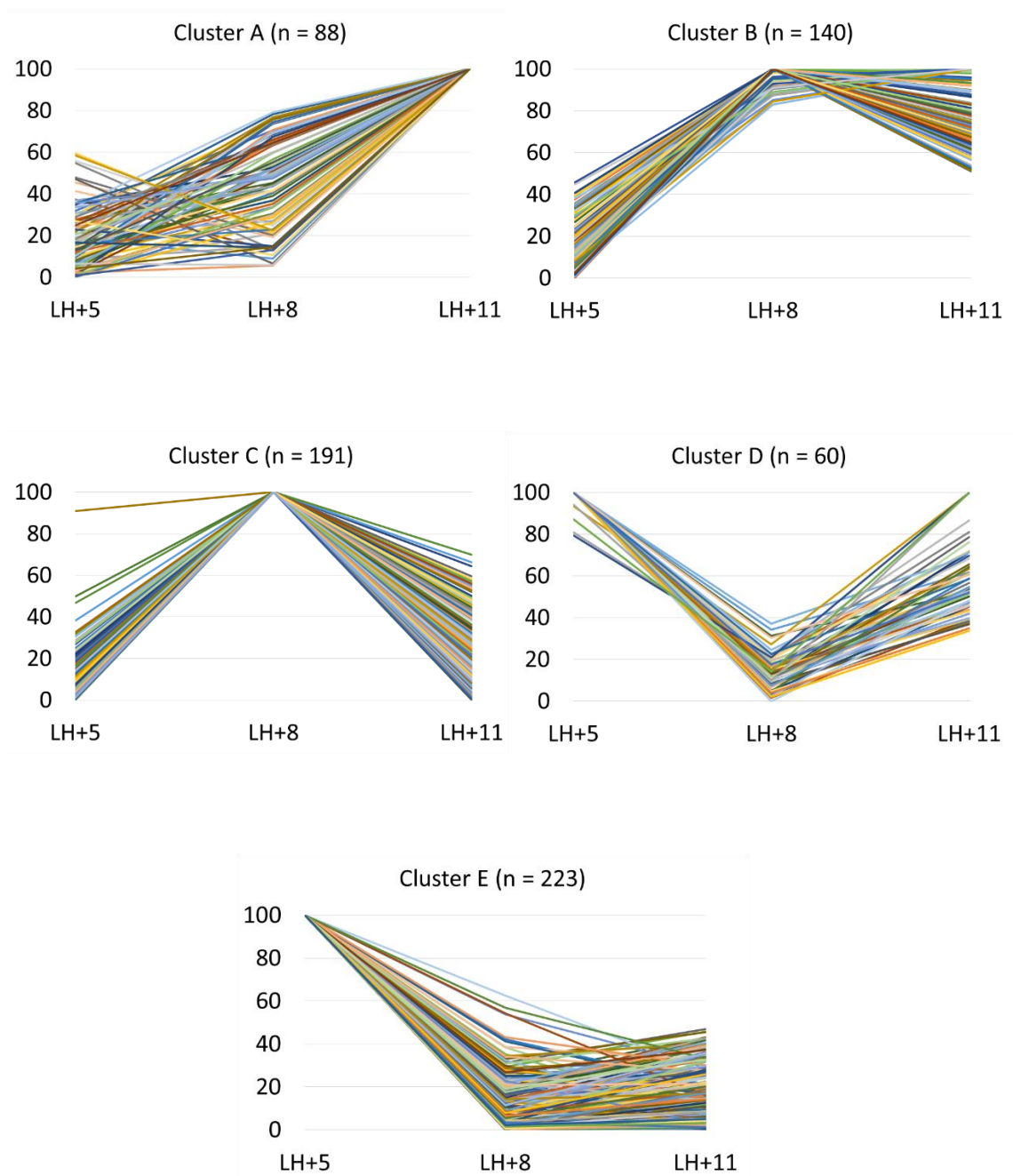
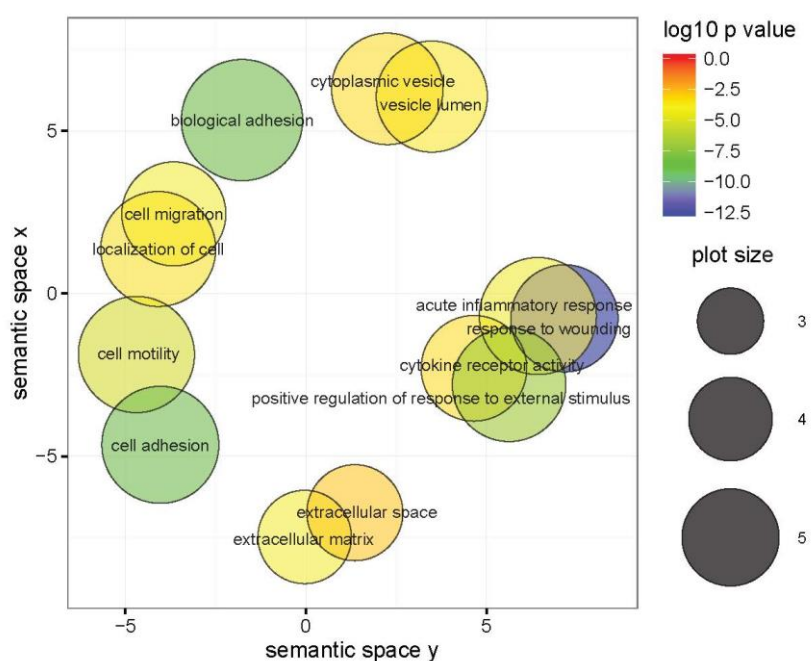


Figure 5.3 Cluster analysis of temporal expression patterns of DEGs in endometrial glands from different time-points in the secretory phase. DEGs in at least one pair-wise comparison using DESeq2 and subjected to K-means cluster analysis using MultiExperiment Viewer v4.9.0 (MeV) uncovered 5 temporal expression patterns.

5.2.2 Gene Ontology Analysis of Glandular Transcriptome, During the Secretory Phase

DEGs in the temporal clusters were submitted to gene ontology (GO) analysis using DAVID Bioinformatics Resources 6.8. Only temporal clusters C and E showed significant GO categories ($P < 0.05$) after Benjamini multiple testing correction. The identified GO categories in these clusters were compatible with processes arising during the acquisition of endometrial receptivity (Supplementary table 5.2 and 5.3). In cluster C, which include DEGs that peaked at the mid-secretory phase, categories such as adhesion, extracellular matrix, inflammation, cytokine signalling and vesicles emerged, and are directly connected to events taking place during embryo implantation (Figure 3.4, top panel). Most of the GO categories engendered from cluster E comprised events connected to cell division (Figure 3.4, bottom panel). The genes in this cluster are downregulated during the mid- and late secretory phases. The findings are consistent with the inhibition of endometrial epithelial proliferation in the period.

Cluster C



Cluster E

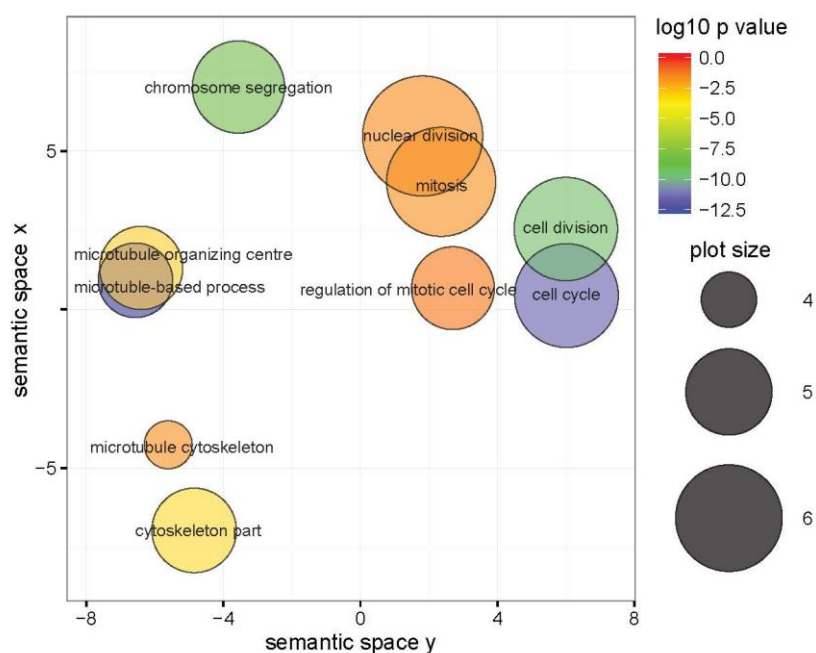


Figure 5.4 GO analysis of temporal clusters C and E. GO terms were obtained by DAVID Bioinformatics Resources 6.8. REVIGO online tool was used to summarize GO categories. Clusters C and E showed significant results following multiple testing correction (Benjamini). Only GO terms with adjusted $P < 0.05$ were plotted. Plot size indicates the frequency of the GO term in the underlying GOA database (Barrell et al. 2009).

5.2.3 Validation of Glandular Transcriptome Profile

To validate my data, I compared the results to two datasets available in GEO Datasets (<https://www.ncbi.nlm.nih.gov/gds>) that examined whole endometrial tissue, using microarray technology. The first study analysed gene expression in the proliferative, early secretory, mid-secretory and late secretory phases of the menstrual cycle (GSE4888, Talbi et al., 2006). The other compared proliferative, early secretory and mid-secretory phase in control women and in women with endometriosis diagnosis (GSE6364, Burney et al., 2007). For my comparisons, I excluded the data from endometriosis patients. The expression of the identified DEGs in the temporal clusters were assessed in both datasets and plotted as heat-maps (Figure 3.5). For each gene, the highest expression level was considered as 100%, and other gene expression levels were calculated based on this assumption. The changes from early to mid-secretory phase were remarkably consistent with our findings. When comparing GSE4888 dataset to our data, during the mid- to late secretory phase, similarities were more evident for clusters B and E. When I focused on specific genes, some of the most upregulated and highest expressed genes in the receptive phase in two previous studies were also significantly induced in my data (Supplementary table 5.4 and 5.5). These findings confirm the importance of the endometrial glands for promotion of a receptive endometrium.

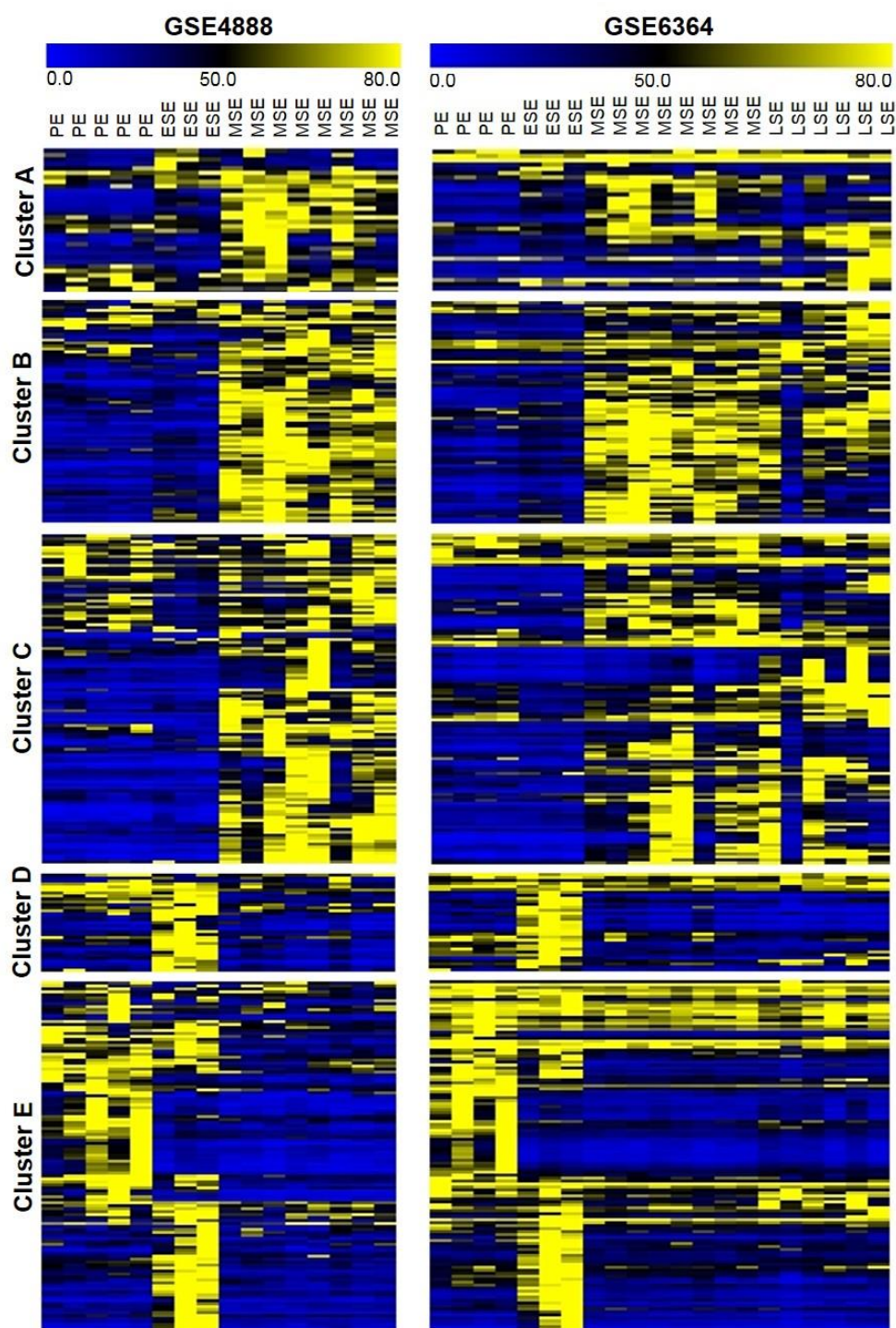


Figure 5.5 Comparison of temporal clusters with external datasets. Heat maps displaying transcription of DEGS from the temporal clusters in two publicly available microarray datasets (GEO Profiles; ID: GSE4888, GSE6364) generated from endometrial tissue in different phases of the menstrual cycle. Transcript levels were expressed in percentage according to the colour bar on top of the heat-maps. The highest transcript levels were rated as 100% for each individual gene. PE: proliferative endometrium; ESE: early secretory endometrium; MSE: mid-secretory endometrium; LSE: late secretory endometrium.

5.2.4 The Endometrial Gland transcriptome in Recurrent Missed Miscarriage

The most profound genomic modifications in endometrial glands were verified during the mid-secretory phase. Therefore, I chose glands from this phase to study the differences between control subjects and women who experienced RMM (Table 3.2). The glands were captured by LMD. Total mRNA was extracted, assessed for integrity (Supplementary table 5.6), amplified, and used for library preparation as previously described. The libraries were sent to the Wellcome Trust Centre for Human Genomics – High Throughput Genomics, Oxford, UK for 100pb paired end sequencing with Illumina HiSeq 4000 system. Data was processed and analysed in collaboration with Dr Pavle Vrljicak. Principal component analysis did not segregate the two groups: control subjects and patients who suffered RMM. Although the wide-genome profile did not show clear difference between the groups, using DESeq2 analysis it was possible to detect 2014 DEGs: 1063 up-regulated in recurrent missed miscarriage cluster, and 951 down-regulated in the same cluster. Gene ontology analysis of the DEGs, using DAVID Bioinformatics Resources 6.8, revealed 25 GO terms out of 60 involved in mitochondrial activity (Figure 3.7; supplementary table 5.7). All the genes within those categories were up-regulated in the RMM group.

Table 5.2 Clinical characteristics of patients from RMM vs control endometrial gland RNA sequencing.

Groups	Control (n=8)	Recurrent missed miscarriage (n=8)	<i>P</i>
Age	35.5 (33-42)	38.5 (29-41)	<i>P</i> = 0.8592
BMI	23.5 (21-36)	24 (22-31)	<i>P</i> = 0.7371
Day of cycle	LH+9 (8-10)	LH+8 (7-9)	<i>P</i> = 0.1251
Miscarriage	0 (0-2)	3.5 (3-8)	<i>P</i> = 0.0002
Live birth	1 (0-2)	0.5 (0-2)	<i>P</i> = 0.8858

Data are presented as median (range)

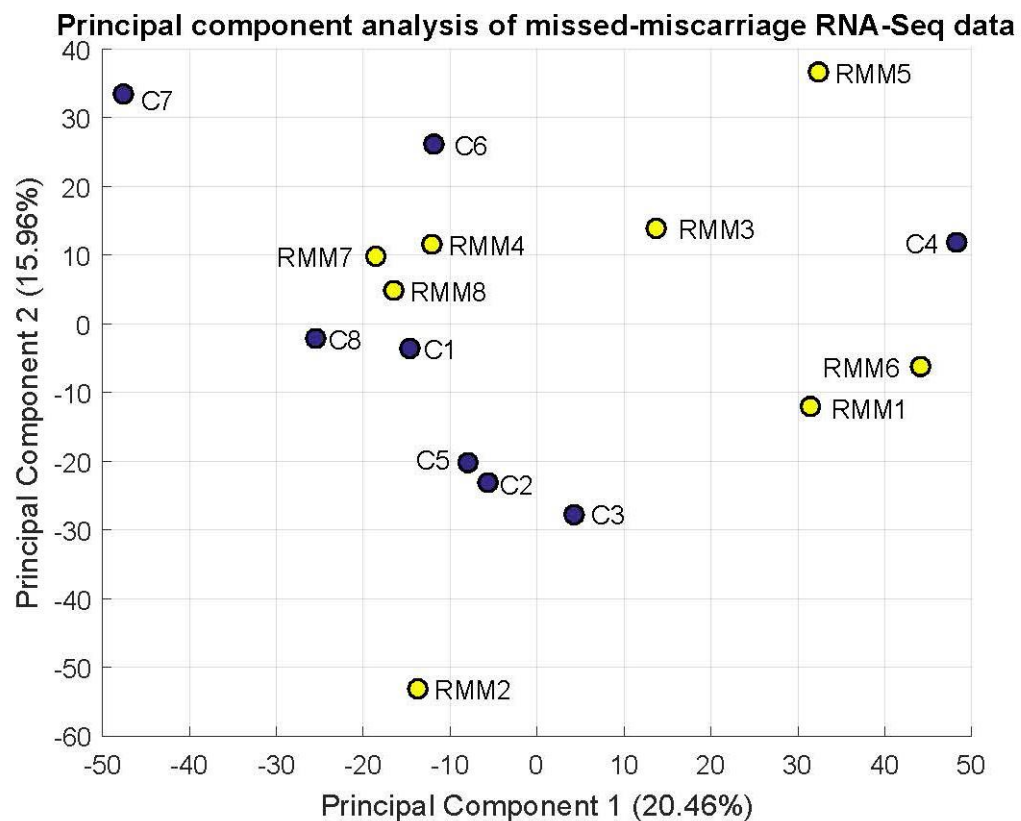


Figure 5.6 Principal component analysis of RNA sequencing data from control subjects and patients who suffered from recurrent missed miscarriage. Principal component analysis of RNA-seq data from control subjects (C) (*n* = 8) and patients who suffered from recurrent missed miscarriage (RMM) (*n* = 8) does not segregate the two groups.

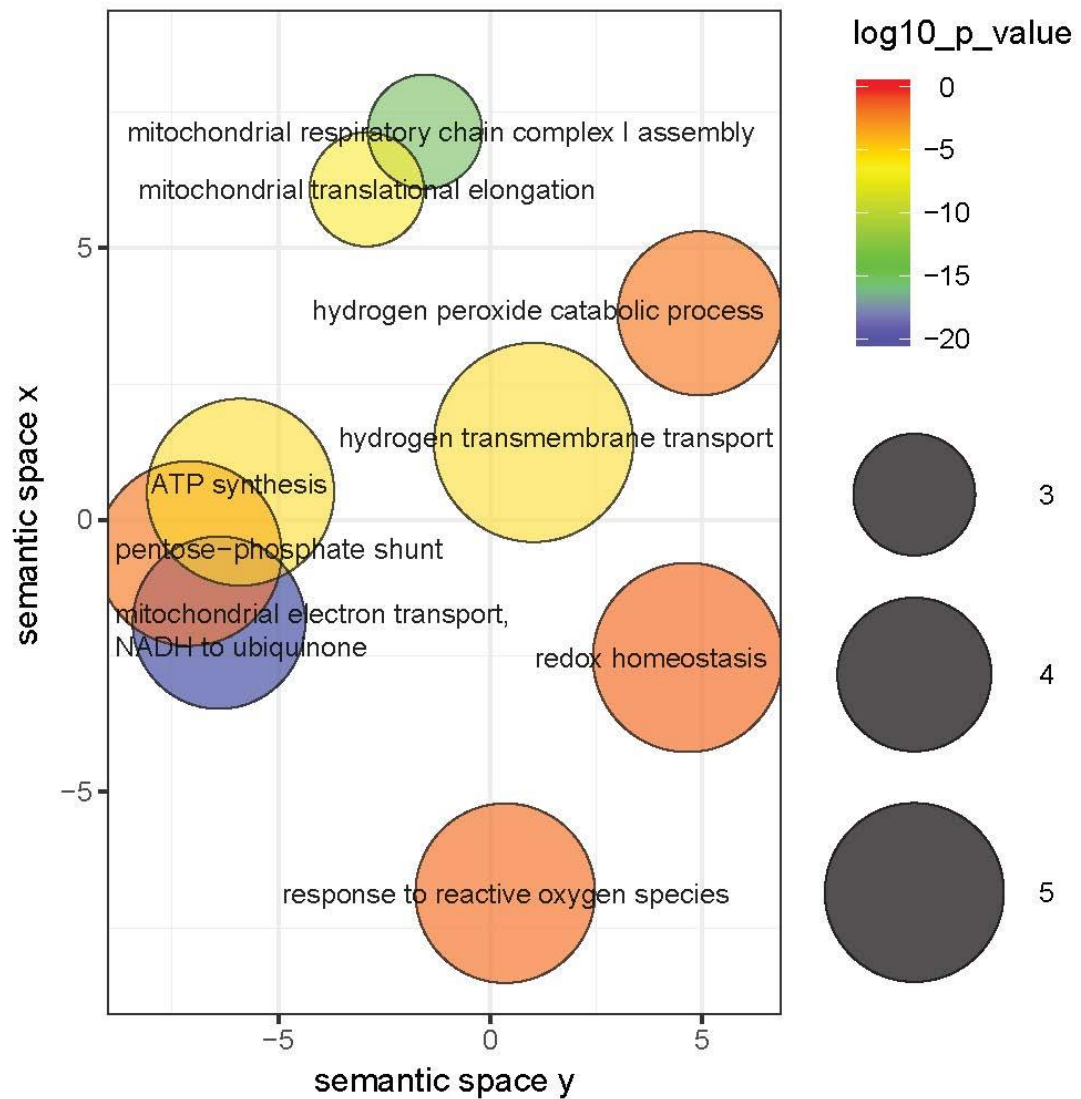


Figure 5.7 Gene ontology (GO) analysis of the RNA sequencing data from control subjects and patients who suffered from recurrent missed miscarriage. GO terms were obtained by DAVID Bioinformatics Resources 6.8. REVIGO online tool was used to summarize GO categories. Only GO terms with adjusted $P < 0.05$ were plotted. Plot size indicates the frequency of the GO term in the underlying GOA database (Barrell et al. 2009).

5.2.5 Validation of RNA Sequencing for Recurrent Missed Miscarriage

Two up-regulated genes (*ALKBH7* and *ZNF319*) and four down-regulated genes (*FAM89A*, *MFAP4*, *TIMP3* and *NID2*) in the recurrent missed miscarriage group were selected for validation of the RNA sequencing data. Independent endometrial glands from 3 control subjects and 3 patients who suffered from recurrent missed miscarriage were captured by LMD. Total mRNA was extracted and used for cDNA synthesis. Expression of the mentioned genes was measured by RT-qPCR. The two up-regulated genes in the recurrent missed miscarriage group in the RNA sequencing data were also significantly up-regulated in the samples used for RT-qPCR. Two out of four of the down-regulated genes in the recurrent missed miscarriage (*FAM89A* and *TIMP3*) were also significantly down-regulated in the samples used for RT-qPCR. Other two genes (*MFAP4* and *NID2*) were also down-regulated, but the differences between the two groups were not statistically significant. Variability in the expression of latter genes in the control group was high, which could account for the lack of significance (Figure 3.8). Genes encoded by mitochondrial DNA were not analysed separately.

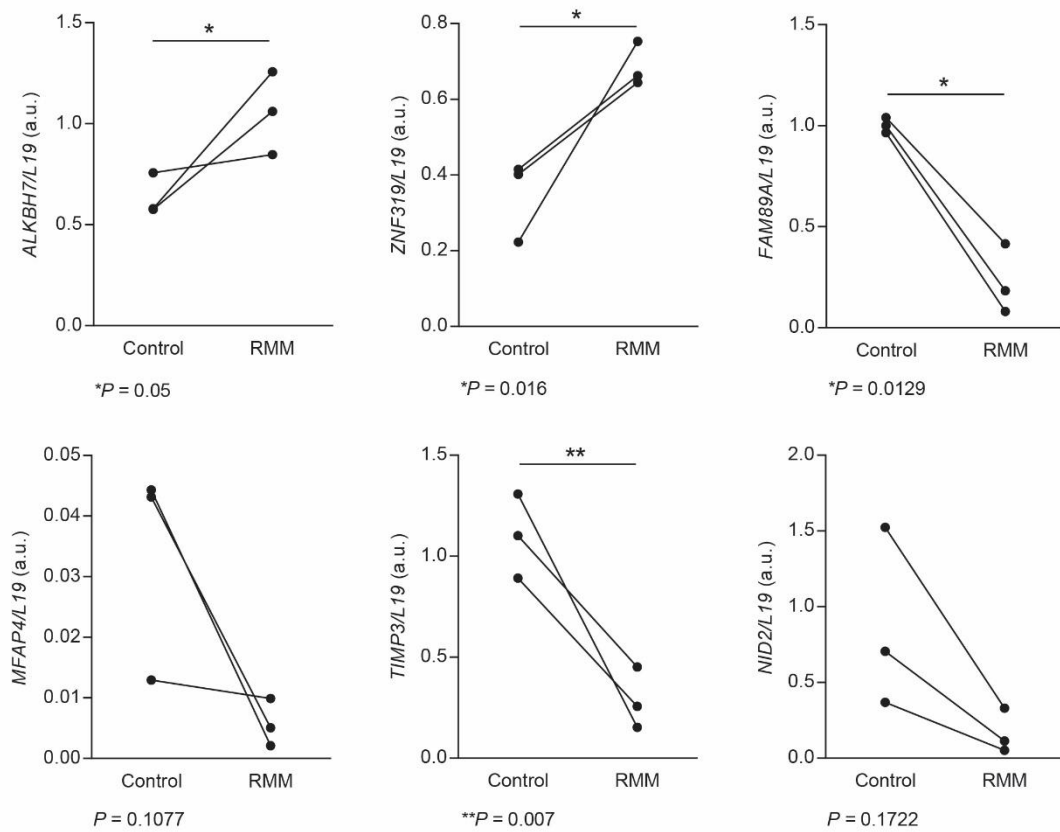


Figure 5.8 Validation of RNA sequencing data from control subjects and patients who suffered from recurrent missed miscarriage. mRNA from endometrial glands from control subjects and patients who suffered from recurrent missed miscarriage was extracted and used for cDNA synthesis. Expression of two up-regulated genes (*ALKBH7* and *ZNF319*) and four down-regulated genes (*FAM89A*, *MFAP4*, *TIMP3* and *NID2*) in recurrent missed miscarriage group from RNA sequencing data was measured. *P* values were calculated using a non-parametric test (Mann-Whitney *U* test).

5.3 Discussion

This is the first analysis of the human endometrial gland transcriptome during the secretory phase, enabling the recognition of a genomic signature for these structures during the receptive period. Our findings were strikingly similar to previous data sets using whole endometrial tissue, suggesting that the most drastic rearrangements during the window of implantation are due to changes in the endometrial glands and not in the luminal epithelium, as is often asserted. The fold change of genes in the mid-secretory phase was strikingly higher in my data, when compared to previous studies. The use of endometrial glands instead of whole endometrium may explain this contrast. In the whole tissue analysis the expression of regulated genes in the glands would be masked by the global expression in the endometrium and its other cell types. The use of microarray and different methods for analysis could also impact on the results. Another difference between my data and the data I used for comparisons was the endometrial dating. In Talbi et al. (2006) and Burney et al. (2007) studies, the endometrium was histologically assessed for defining the phases. Díaz-Gimeno et al. (2005) also used the LH surge as a reference, but they chose LH+2 and LH+7, labelled as early and mid-secretory endometrium, respectively. This may explain the less pronounced changes observed from mid- to late secretory phase in my data. Some authors include LH+11 day in the window of implantation (Achache and Revel, 2006, Fatemi and Popovic-Todorovic, 2013, Zhang et al., 2013).

The GO terms that emerged in the mid-secretory phase are consistent with the events taking place during embryo implantation. The main changes observed in the plasma membrane of endometrial epithelial cells, during the window of implantation, are the expression of adhesion molecules (Singh and Aplin, 2009). A remodelling of the extracellular space and ECM occurs in order to accommodate the implanting blastocyst (Salamonsen and Nie, 2002). Recruitment of dendritic cells and macrophages are a common feature of response to wounding and uterine receptivity

(Lee et al., 2011). An acute inflammatory response takes place in the initial phase of implantation, involving intense cytokine signalling (Dekel et al., 2014). An increase in cytoplasmic vesicles in endometrial gland cells can be detected during the active period of gland secretion (Hempstock et al., 2004). The GO terms yielded from temporal cluster C match all these phenomena. DEGs in this cluster displayed a peak in the mid-secretory phase. By contrast, DEGs in the cluster E are down-regulated after the early secretory phase. The GO terms produced from this cluster are all related to cell division. P4 levels increase along the first half of luteal phase. Endometrial epithelial proliferation is inhibited by P4 (Kurita et al., 1998).

The endometrial gland transcriptome of patients who suffered from RMM suggested possible affected cell functions in this condition. Out of 60 GO terms generated, 25 were somehow involved in mitochondrial activity. All the genes in these categories were up-regulated in the recurrent miscarriage group. ATP synthases (*ATP5I*, *ATP5G3*, *ATP5L*, *APT5E*, *ATP5J2*, *MT-ATP6*) produce ATP from ADP and inorganic phosphate (Wittig et al., 2009, Pagadala et al., 2011). Cytochrome c oxidases (*MT-CO3*, *MT-CO2*, *COX5A*, *COX5B*, *COX6A1*, *COX7A2*, *COX7B*, *COX8A*) and mitochondria encoded-NADH dehydrogenase (*MT-ND2*, *MT-ND3*, *MT-ND4*) are directly involved in oxidative phosphorylation and respiratory electron transport chain (Weiss et al., 1991, Abril et al., 2008, Wikström, 2010). COA3 regulates the assembly of cytochrome C (Ostergaard et al., 2015). Solute carriers family 25 A (*SLC25A1*, *SLC25A14*, *SLC25A11*, *SLC25A22*) and mitochondria outer and inner translocase complexes control the transport of small metabolites and proteins through the mitochondria membranes (Hönlinger et al., 1996, Rehling et al., 2004, Gutiérrez-Aguilar et al., 2013, Palmieri, 2013). *TXN2*, *PRDX5* and *OSER1* have antioxidant roles (Nguyễn-nhu and Knoop, 2003, Yuan et al. 2004, Pérez et al., 2007, Zhang et al., 2007, Barl et al., 2016). *ALKBH7*, *MTCH1* and *APOPT1* regulate mitochondria-mediated apoptosis (Lamarca et al., 2007, Fu et al., 2013, Melchionda et al., 2014,

Wang et al., 2014). I postulated that an imbalance in gland-cells energy spending would lead to premature exhaustion of the endometrial glands, deficient histiotrophic support and, as a consequence, to foetal underdevelopment and demise. I have demonstrated reduced clonogenicity of endometrial gland cells in patients with recurrent missed miscarriage (Chapter 2). Mitochondria density and activity are low in stem-cells and increase upon differentiation (Mandal et al., 2010, Prigione et al., 2010). Therefore higher expression of mitochondria related genes in recurrent missed miscarriage samples could simply reflect the lower amount of clonogenic cells in the glands of these patients. These possibilities need to be further explored.

Chapter 6

Final Discussion

6.1 Isolation, Culture and Reprogramming of HEEC

The problem of cell cycle arrest, disturbing proliferation of HEECs *in vitro* was corroborated by my research. Once cells were isolated from the tissue, plated as monolayers, and cultured in non-specific medium, they exhibited protracted growth and developed acute senescence compared to HESCs. Furthermore, stimulation with ovarian steroids and embryonic cues (trypsin and hCG) did not evoke differentiation responses. Conditional reprogramming, targeting cytoskeleton remodelling (ROCK inhibitor) and increase of TERT activity (conditioned medium from irradiated fibroblasts) improved HEEC proliferation and partially rescued them from acute senescence. This approach enabled expansion and freezing, allowing the establishment of stocks of cells from patients with specific clinical characteristics or conditions.

Reprogrammed HEECs remained unresponsive to sex steroids and embryo-derived signals, however, decidualized stromal secreted factors elicited differentiation, enhanced proliferation, and reduced senescence. Which soluble factors from differentiated stroma induced the responses in the HEECs is still a matter of speculation, and future investigation could unravel another crucial aspect of the epithelial-stromal cross-talk. An obvious candidate would be PRL, since it is a major protein secreted by the decidualized endometrial fibroblasts. However, no response was observed when HEECs were exposed to this hormone. Transcriptome, proteome and secretome analysis could provide a list of decidua-secreted proteins to be further investigated. Some of these studies detected noteworthy functional annotations such as cell cycle regulation, cytoskeleton remodelling, and growth factor and cytokine signalling (Takano et al., 2007, Garrido-Gomez et al., 2010, Paule et al., 2010). An interesting observation was that conditioned medium from HESCs decidualized for 4 and 10 days trigger a similar response. Gene expression during decidualization is dynamic, with pronounced time-dependant changes. Salker et al. (2012) identified a

dramatic shift in the expression of several inflammatory mediators between HESCs decidualized for 2 or 8 days. Lucas et al. (2015) also observed great variability within 18 secreted proteins (cytokines, chemokines and growth factors) from HESCs decidualized for 2, 4 or 8 days. These changes could help selecting the most likely HEEC differentiation factors, through elimination of those that show marked variation between early and late decidualization period. Some factors expressed by the stroma, such as HGF, FGF7, FGF10, WNT4, WNT5A, are involved in endometrial adenogenesis and development, and may be implicated in the gland differentiation observed with decidualized HESC conditioned medium (Taylor et al., 2001, Cooke et al., 2013).

Basal adenylyl cyclase activity in the endometrium is increased during the secretory phase, and this activity is more highly induced upon PGE₂ treatment in the same phase compared to proliferative phase (Tanaka et al., 1993). P4 enhances PGE₂-promoted cAMP production in HESCs (Houserman et al., 1989). This accounts for the extremely high tissular content of cAMP in the luteal-phase endometrium (Bergamini et al., 1985). Considering these observations, it is reasonable to cogitate that stroma-derived cAMP could be a possible factor driving epithelial differentiation, during decidualization. Indeed, when later, I treated endometrial gland organoids with this second messenger an up-regulation of glycodeilin gene was observed.

The data corroborate the importance of the epithelial-stromal cross-talk in the endometrial function, and indicated a novel role for the decidual cells in the regulation of epithelial proliferation, differentiation and senescence.

6.2 Three Dimensional Culture of HEECs

Cells in monolayer culture do not polarize properly, having a limited space to interact with neighbouring counterparts (Baker and Chen, 2012). This problem is even more pronounced when using epithelial glandular cells (Rodriguez-Boulán and Macara,

2014). Trafficking of molecules must be organized to allow secretion through the lumen and cross-talk with the underlying compartment, and polarity is essential for this coordination. Furthermore, receptors and ion channels are usually located in the apical domain, which is also crucial for signal transduction (Kaplan et al., 2009). Based on these facts I conjectured that HEEC monolayers did not respond to differentiation stimuli due to deficient polarization, which prompted me to develop a method for 3D culture.

HEEC spheroids were relatively easy to produce, however it is not so simple to handle them. Due to the limited volume of media in the hanging drops, the structures must be soon transferred to a plates or dishes that holds larger liquid volume. Rupture of the structures during the transfer were frequent, and a large number of spheroids were required to extract a reasonable mRNA amount. Another risk was the possibility of deficient perfusion of cells in the core of spheroids (Edmondson et al., 2014). Two approaches could be used to circumvent this problem. First, there are viability assays for spheroids described in the literature (Bell et al., 2016, McMillan et al., 2016). Assessing the viability, it would be possible to optimize the number of cells used to produce the spheroids, although risking to further reduce the quantity of retrieved mRNA. Second alternative is the use of microcarrier cultures, which are based on the use of microbeads for the cells to attach. The core is occupied by the beads and the cells are spread onto the surface. Many beads can be added in the dish at the same time, enabling work with larger number of cells (Goh et al., 2013).

Another modality of 3D culture attempted was the polymer scaffolds. This work is still in progress, in order to optimize the material, the pore size and the chemoattractant used for coating the scaffold. I have demonstrated that the cells grow in a 3D disposition, however polarity is still to be tested with the staining of some subcellular structures, such as microvilli and adhesion molecules from the lateral and basal domains (Eritja et al., 2010). There is a strong possibility that the cells are laying in a

configuration more akin to LE than GE, based on the staining performed so far. Expansion medium could be tested to induce a glandular arrangement in the scaffold. An interesting alternative to the synthetic scaffolds has just been explored by our group: the use of decellularized scaffolds. Fragments of tissue are submitted to physical (agitation, thermal shock, ultrasound and hydrostatic pressure), chemical (detergents, solvents, ionic solutions) or enzymatic process to remove the cells and preserve the ECM (Tapias and Ott, 2014). The ECM scaffold can be then used to harbour new cells. Evidence has also indicated the advantage of the utilization of scaffolds coupled with microfluidic devices (Tehranirokh et al., 2013). Employing a system of channels and chambers, it is possible to delivery substrates, nutrients and reagents in a controlled way, improving the cell / tissue microenvironment.

Using a modified adult stem-cell medium it was possible to establish endometrial gland organoids with high efficiency. These organoids could be derived from individual patients, expanded and be used for functional studies, enabling personalized study of uterine glands *in vitro*. Some improvements still must be done to further purify and characterize these structures. For example, the presence of a HEEC monolayer, underlying the Matrigel drop, possibly masks organoid responses to the differentiation cues applied. The clear more pronounced induction of *PAEP* in the organoids in OFE assays strongly suggests that. In this assay, a lower number of cells is seeded in a small volume of Matrigel, and the presence of HEECs attached to the well bottom is scarce or inexistent. Optimization of the seeding cell number, the volume of basement membrane matrix, and the harvesting of cells may solve this problem.

Glycogen production did not differ between control and differentiated organoids. Once more, the presence of the underlying HEEC monolayer may be accounted for hiding the differences between the two groups. Another possibility is a technical problem with the employed assay. It ultimately measures glucose content in the lysate, after

digestion of glycogen. Reducing substances and high protein content in the samples may interfere with the observed results. The organoids are retrieved from Matrigel using Cell Recovery Solution and several washes, however it is impossible to totally remove the matrix. The composition of this product is variable, but a high amount of protein is always present (Kleinman et al., 1986, Vukicevic et al., 1992). Therefore it is not possible to rule out the chance of the Matrigel or even the medium components influencing the results of the assay. An interesting alternative is the periodic-acid Schiff staining. This method is used for detecting glycogen in tissue and could be used with the organoids, provided that sections of intact organoids are obtained.

The possibility of studying endometrial glands *in vivo* creates opportunities to further investigate their role in stromal decidualization, endometrial receptivity and immune regulation of maternal tolerance to the foetus, or even to unravel new functions for these glands. Co-culture with stromal cells or blastocysts allows exploration of the exchange of signals between these endometrial compartments, and between the implanting embryo and the endometrium. Research on several clinical conditions, such as endometriosis, RPL, endometrial hyperplasia and endometrial cancer, can also profit with the establishment of patient-specific gland organoids. The discovery of specific gland defects in RPL and endometriosis can aid in the development of a treatment. Identified prognostic factors for hyperplasia clinical evolution, i.e. the chance of developing an invasive cancer, and for endometrial adenocarcinoma progression may guide therapeutic choices and even preventive measures.

The use of 3D culture methods for investigation of endometrium is a promising tool in the advance of our knowledge on this intriguing and complex tissue. Furthermore, the study of uterine glands *in vitro* enables a deeper understanding of the role of these structures in reproduction and in pathological conditions.

6.3 Transcriptome of Endometrial Glands in the Mid-Secretory Phase

The endometrial gland transcriptomic signature during the WOI was markedly concordant with previous whole endometrial tissue studies. This similarity suggests that the transformations taking place during the receptive phase are mainly due to changes in the GE. Actually, it is not possible at the moment to affirm that the same behaviour is not present in the LE. Remarkable differences have been demonstrated between these two portions of the endometrial compartment (Demir et al., 2002, Niklaus and Pollard, 2006), besides, several of the top most up-regulated genes in mid-secretory phase, such as *PAEP*, *DPP4*, *CXCL14*, *DEFB1*, *SLP1* are chiefly expressed by GE (Sepällä et al., 2002, Imai et al., 1992, Mokhtar et al., 2010, Das et al., 2007, King et al., 2000). The significant GO terms that emerged from two clusters of my data are consistent with phenomena observed during the acquisition of endometrial receptivity and blastocyst implantation, corroborating the role of endometrial glands in the preparing the endometrium to accommodate the embryo.

6.4 Endometrial Glands in RMM

Due to the essential role of uterine glands to provide histiotrophic support to the foetus during early pregnancy I postulated that defects in these epithelial compartment would be implicated in cases of RMM. In such cases, the underdeveloped embryo cannot be identified or a protracted foetal development is followed by demise (Sur and Raine-Fenning 2009).

Clonogenicity of endometrial gland cells from RMM cases was significantly decreased compared to that of control subjects, as demonstrated by the OFE assay. The number of organoids formed after seeding at clonal density was lower and the diameter was usually smaller in the RMM group. I postulate that these progenitor cells would fail to

expand when demanded, reducing the decidual gland density at the maternal-foetal interface, leading to deficient histiotrophic support and eventually foetal death.

When analysing the RNA sequencing data from control subjects and RMM cases, principal components did not split the two groups, nevertheless a high number of DEGs were detected by DeSeq2 analysis. GO analysis produced several categories involved in mitochondrial functions, such as ATP synthesis, free-radical scavenging and apoptosis. All the DEGs were up-regulated in the RMM group. Gland secretion is a bioenergetically demanding activity, involving protein translation, assembly, folding, packaging, trafficking and exocytosis, requiring high efficiency from the mitochondrial machinery. I postulate that in RMM cases, the uterine glands develop premature exhaustion, before the establishment of the haemochorial support by placenta, due to mitochondrial defect. Since these organelles are also involved in regulation of apoptosis and prevention of excessive oxidative stress, it might be that impairment in these functions could lead to uncontrolled DNA damage and cell death in the placenta. The finding of higher expression of mitochondria-associated genes in the RMM cases may be related to my previous observation of decreased gland clonogenicity in such condition. Stem cells exhibit lower mitochondrial density and activity (Mandal et al., 2010, Prigione et al., 2010). RMM samples would harbour less stem-like cells, thereby presenting higher mitochondrial contents. Analysis of mitochondrial activity in endometrial gland organoids could further explore the role of the organelle in RMM. The Seahorse XF Cell Mito Stress Test (Seahorse Bioscience) measures direct cell oxygen consumption, estimating parameters of mitochondria function, such as basal respiration, ATP-linked respiration, H⁺ (proton) leak, maximal respiration, spare respiration and non-mitochondrial respiration (Isono et al., 2016). Ultrastructural analysis of the cells in the organoids could allow direct visualization of mitochondria, thereby enabling to analyse mitochondria density, shape and size. Curiously, Armstrong et al. (1973) described a giant mitochondria found exclusively

in endometrial gland cells, from day 13 to day 22 of the menstrual cycle. These organelles were frequently connected to the endoplasmic reticulum. Dockery et al. (1993) observed lower density and smaller size of mitochondria in infertile patients in the early secretory phase (LH +4). The same group detected a decrease in mitochondrial size at LH+5 in patients receiving the anti-progesterone drug, mifepristone (Dockery et al., 2007).

Glandular defects are most likely associated with recurrent empty sacs or malformed foetus with normal karyotypes. In these cases, the deficient supply of nutrients (amongst them retinoic acid) and growth factors, by impaired endometrial glands, would lead to defective organogenesis, even though no chromosomal abnormality is present. Despite the reasonable number of samples, my results emerge only as a starting point for future investigation. It also pioneers the approach of the patient outside the pregnancy, being possible to establish a causal association. Larger sample sizes and stringent selection of cases and controls will help to validate or reject the observations.

Appendices

Supplementary table 5.1: RNA integrity number (RIN) and 28s/18s rRNA ratio of the samples used in LH-timed endometrial gland assay

Sample ID	RIN	28s/18s rRNA ratio
ES1	6.2	1.2
ES2	6.8	1.2
ES3	7.2	1.3
MS1	6.9	1.2
MS2	7.0	1.3
MS3	7.0	1.2
LS1	6.8	1.3
LS2	6.8	1.2
LS3	6.7	1.0

Supplementary table 5.2: GO terms and categories for temporal cluster C.
Categories and terms were only included if $P < 0.05$.

Category	Term	Adjusted P
GO:0044421	extracellular region part	9.72E-07
GO:0005576	extracellular region	3.44E-06
GO:0009611	response to wounding	0.001993
GO:0005615	extracellular space	0.001696
GO:0002526	acute inflammatory response	0.020448
GO:0007155	cell adhesion	0.028643
GO:0022610	biological adhesion	0.021954
GO:0004896	cytokine receptor activity	0.04803
GO:0050729	positive regulation of inflammatory response	0.039032
GO:0031093	platelet alpha granule lumen	0.028022
GO:0060205	cytoplasmic membrane-bounded vesicle lumen	0.029373
GO:0005604	basement membrane	0.027283
GO:0044420	extracellular matrix part	0.023538
GO:0031983	vesicle lumen	0.02183
GO:0005578	proteinaceous extracellular matrix	0.020632
GO:0031410	cytoplasmic vesicle	0.02256
GO:0031012	extracellular matrix	0.02948
GO:0005577	fibrinogen complex	0.027822
GO:0031982	vesicle	0.026376
GO:0031091	platelet alpha granule	0.026035
GO:0032103	positive regulation of response to external stimulus	0.031942
GO:0042060	wound healing	0.036671

Supplementary table 5.3: Some of GO categories and terms for temporal cluster E. Categories and terms were only included if adjusted $P < 0.05$.

Category	Term	Adjusted P
GO:0000279	M phase	1.1E-10
GO:0000280	nuclear division	1.28E-10
GO:0007067	mitosis	1.28E-10
GO:0000087	M phase of mitotic cell cycle	1.2E-10
GO:0048285	organelle fission	1.36E-10
GO:0007049	cell cycle	1.39E-09
GO:0022402	cell cycle process	1.75E-09
GO:0022403	cell cycle phase	2.21E-09
GO:0000278	mitotic cell cycle	9.3E-09
GO:0051301	cell division	4.91E-08
GO:0007059	chromosome segregation	1.15E-07
GO:0015630	microtubule cytoskeleton	4.81E-07
GO:0005819	spindle	4.88E-06
GO:0007346	regulation of mitotic cell cycle	9.2E-06
GO:0000793	condensed chromosome	7.85E-06
GO:0044430	cytoskeletal part	2.5E-05
GO:0005694	chromosome	6.29E-05
GO:0000777	condensed chromosome kinetochore	8.93E-05
GO:0044427	chromosomal part	8.78E-05
GO:0043228	non-membrane-bounded organelle	8.99E-05
GO:0043232	intracellular non-membrane-bounded organelle	8.99E-05
GO:0000779	condensed chromosome, centromeric region	0.000144
GO:0000775	chromosome, centromeric region	0.000139
GO:0005856	cytoskeleton	0.000213
GO:0051726	regulation of cell cycle	0.001306
GO:0000776	kinetochore	0.000303
GO:0010564	regulation of cell cycle process	0.002878
GO:0000070	mitotic sister chromatid segregation	0.003354
GO:0000819	sister chromatid segregation	0.003588
GO:0000075	cell cycle checkpoint	0.004135
GO:0005815	microtubule organizing center	0.001249
GO:0005813	centrosome	0.002022

Supplementary table 5.4: Up-regulated genes from early to mid-secretory phase in natural menstrual cycle. Comparison of the present study with two similar studies.

Gene name	Present study (Fold change)	Talbi et al., 2006 (Fold change)	Díaz-Gimeno et al., 2005 (Fold change)
DPP4	263.47	12.34	7.72
GPX3	217.90	32.52	35.49
COMP	203.97	14.22	30.95
GAST	167.2541	6.44	5.00
AOX1	152.87	5.26	4.82
C4BPA	151.39	6.67	13.14
CXCL14	132.06	39.13	14.02
PAEP	119.93	23.55	31.43
LAMB3	96.32	6.07	11.32
CP	77.86	4.93	6.34
LIF	77.30	4.11	15.03
TSPAN8	73.06	11.57	12.90
CD55	57.23	8.62	22.47
SLC15A1	50.61	13.29	5.59
ABCC3	46.54	2.35	5.98
APOD	37.75	3.55	4.32
TCN1	31.20	9.58	14.76
NNMT	29.74	1.49	7.74
SERPING1	22.91	4.82	5.16
MAOA	21.9	3.99	9.39
DEFB1	19.9	8.62	4.17
MT1H	17.83	1.70	3.12
SLC1A1	17.57	21.66	17.57
GBP2	17.46	2.94	3.14
MUC16	15.24	7.61	8.01
CRISP3	13.32	3.90	5.09
C3	12.10	4.35	4.67
VCAN	11.40	4.37	3.24
ANXA4	10.25	4.09	4.27
SLPI	9.90	4.01	4.39
SYNE2	9.30	4.22	3.52
ARID5B	8.36	4.17	4.19

Supplementary table 5.5: Down-regulated genes from early to mid-secretory phase in natural menstrual cycle. Comparison of the present study with two other similar studies.

Gene name	Present study (Fold change)	Talbi et al., 2006 (Fold change)	Díaz-Gimeno et al., 2005 (Fold change)
ALPL	-60.48	-5.75	-4.44
PLA1A	-46.93	0.98	-3.42
LRP4	-45.92	-9.64	-5.87
CTNNA2	-32.78	-13.35	-8.02
OPRK1	-24.41	-19.40	-3.17
CSRP2	-21.69	-10.65	-9.62
HLA-DOB	-21.05	-13.27	-11.06
SLC15A2	-19.55	-8.31	-8.44
LRRC17	-19.30	-6.39	-4.64
ATP6V0E2	-19.30	-5.33	-9.82
EDN3	-16.51	-4.09	-5.30
NR4A2	-15.15	-4.53	-7.05
SORD	-14.32	-4.38	-3.21
BUB1B	-13.40	-1.52	-3.05
CCNB2	-11.61	-1.29	-3.23
GALNT12	-11.00	-4.33	-4.85
PRKCQ	-9.50	-2.84	-3.08
IDH1	-9.46	-5.31	-3.68
NDRG2	-8.92	-3.29	-3.40
KHDRBS3	-8.46	-3.61	-3.51
SOX17	-7.90	-4.76	-4.24
CKB	-7.79	-2.51	-3.61
CREB3L1	-7.56	-4.29	-3.77
BARD1	-7.55	-3.53	-3.47
ANK3	-7.06	-5.31	-4.79
STEAP4	-6.86	-5.47	-3.04
DFNB31	-6.56	-4.94	-3.54
HSD11B2	-5.63	-3.01	-4.03
MSX1	-5.10	-3.49	-3.26
MMP26	-4.74	-3.45	-4.66
OFD1	-3.41	-3.92	-3.44

Supplementary table 5.6: RNA integrity number (RIN) and 28s/18s rRNA ratio of the samples used in missed miscarriage assay.

Sample ID	RIN	28s/18s rRNA ratio
C1	6.9	1.0
C2	6.6	1.2
C3	6.4	1.0
C4	7.8	1.1
C5	6.0	1.0
C6	6.3	1.0
C7	6.4	1.0
C8	7.2	1.1
C9	6.9	1.1
RMM1	6.4	1.1
RMM2	6.8	1.3
RMM3	6.6	1.0
RMM4	6.1	1.1
RMM5	7	1.4
RMM6	7.3	1.3
RMM7	7.2	1.1
RMM8	5.7	1.1
RMM9	7.1	1.2

Supplementary table 5.7: GO categories and terms of recurrent missed miscarriage versus control subjects RNA sequencing data. Categories and terms were only included if adjusted $P < 0.05$.

Category	Term	Adjusted P
GO:0005743	mitochondrial inner membrane	1.00882E-25
GO:0006120	mitochondrial electron transport, NADH to ubiquinone	1.47052E-21
GO:0005747	mitochondrial respiratory chain complex I	4.74348E-20
GO:0008137	NADH dehydrogenase (ubiquinone) activity	9.02256E-18
GO:0032981	mitochondrial respiratory chain complex I assembly	6.10612E-16
GO:0003735	structural constituent of ribosome	1.12726E-09
GO:0006123	mitochondrial electron transport, cytochrome c to oxygen	1.5118E-09
GO:0004129	cytochrome-c oxidase activity	1.97895E-09
GO:0006412	translation	3.28961E-06
GO:0070125	mitochondrial translational elongation	6.84563E-07
GO:0005762	mitochondrial large ribosomal subunit	7.19562E-07
GO:0070126	mitochondrial translational termination	8.07507E-07
GO:1902600	hydrogen ion transmembrane transport	2.12992E-06
GO:0006754	ATP biosynthetic process	2.47538E-06
GO:0005753	mitochondrial proton-transporting ATP synthase complex	2.17475E-05
GO:0005840	ribosome	0.003633511
GO:0005751	mitochondrial respiratory chain complex IV	0.000102057
GO:0042776	mitochondrial ATP synthesis coupled proton transport	0.000154348
GO:0019058	viral life cycle	0.000306093

GO:0006094	gluconeogenesis	0.000553001
GO:0006614	SRP-dependent cotranslational protein targeting to membrane	0.000937854
GO:0000184	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	0.002383755
GO:0015078	hydrogen ion transmembrane transporter activity	0.000154348
GO:0022625	cytosolic large ribosomal subunit	0.003589483
GO:0048029	monosaccharide binding	0.005821039
GO:0003899	DNA-directed RNA polymerase activity	0.005857744
GO:0006364	rRNA processing	0.006877368
GO:0005665	DNA-directed RNA polymerase II, core complex	0.00707246
GO:0050434	positive regulation of viral transcription	0.007731698
GO:0042744	hydrogen peroxide catabolic process	0.009941164
GO:0036258	multivesicular body assembly	0.010299137
GO:0006098	pentose-phosphate shunt	0.010477153
GO:0039536	negative regulation of RIG-I signaling pathway	0.010743627
GO:0019083	viral transcription	0.011011321
GO:0031902	late endosome membrane	0.011362093
GO:0006368	transcription elongation from RNA polymerase II promoter	0.011630563
GO:0006370	7-methylguanosine mRNA capping	0.013396146
GO:0001054	RNA polymerase I activity	0.013814991
GO:0039702	viral budding via host ESCRT complex	0.014011183
GO:0000276	mitochondrial proton-transporting ATP synthase complex, coupling factor F(o)	0.014052432
GO:0098641	cadherin binding involved in cell-cell adhesion	0.017618238

GO:0005736	DNA-directed RNA polymerase I complex	0.017676902
GO:0006413	translational initiation	0.018523761
GO:0051537	2 iron, 2 sulfur cluster binding	0.019506866
GO:0031145	anaphase-promoting complex-dependent catabolic process	0.021562589
GO:0016197	endosomal transport	0.02443826
GO:0061621	canonical glycolysis	0.024971643
GO:0000302	response to reactive oxygen species	0.026270112
GO:0005868	cytoplasmic dynein complex	0.026337883
GO:0098609	cell-cell adhesion	0.029300056
GO:0005913	cell-cell adherens junction	0.030422093
GO:0051287	NAD binding	0.032811347
GO:0000398	mRNA splicing, via spliceosome	0.034105971
GO:0042542	response to hydrogen peroxide	0.034439994
GO:0051436	negative regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	0.034715987
GO:1902041	regulation of extrinsic apoptotic signaling pathway via death domain receptors	0.035568928
GO:0000920	cell separation after cytokinesis	0.041337226
GO:0006283	transcription-coupled nucleotide-excision repair	0.042083084
GO:0045454	cell redox homeostasis	0.047517331
GO:0051437	positive regulation of ubiquitin-protein ligase activity involved in regulation of mitotic cell cycle transition	0.047517331

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Publications

Isolation and Primary Culture of Various Cell Types from Whole Human Endometrial Biopsies

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[Abstract] The isolation and primary culture of cells from human endometrial biopsies provides valuable experimental material for reproductive and gynaecological research. Whole endometrial biopsies are collected from consenting women and digested with collagenase and DNase I to dissociate cells from the extracellular matrix. Cell populations are then isolated through culturing, filtering and magnetic separation using cell-surface antigen markers. Here we provide a comprehensive protocol on how to isolate and culture individual cell types from whole endometrial tissues for use in *in vitro* experiments.

[Background] The human endometrium is the inner most mucosal layer of the uterus. It consists of a columnar epithelium and basal stromal layer that undergoes cyclical regeneration, growth and transformation in response to circulating hormones. The differentiation of the endometrial lining into a glandular secretory phenotype provides a hospitable environment for blastocyst implantation and successful pregnancy. In the absence of pregnancy this layer is shed, leading to menstruation. The isolation and culture of cells from human endometrial biopsies allows for *in vitro* functional assessment and the study of cell characteristics in relation to patient outcomes. The isolation and culture of endometrial cells is an invaluable research model to investigate many aspects of gynaecological and obstetrical medicine including infertility, implantation failure, recurrent miscarriage and menstrual disorders. Whole human endometrial biopsies contain human endometrial stromal cells (HESCs), luminal and glandular endometrial epithelial cells (HEECs), red blood cells and a mixed population of immune cells. HESCs can be easily and inexpensively isolated from whole biopsies and actively proliferate in culture for up to 5 passages without significant change in their growth dynamics. This provides a large window of opportunity for experimental analysis. Furthermore, within dissociated HESCs there is a sub-population of perivascular progenitor mesenchymal stem-like cells that can be isolated using the perivascular-specific antigen SUSD2 and its cognate antibody W5C5. Here we provide in detail an updated and expanded protocol from those published previously (Masuda *et al.*, 2012; Chen and Roan, 2015) to describe steps in isolating and culturing different cell types from whole human endometrium. We provide further information on biopsy collection, detailed protocols for isolation of progenitor cells and additional procedures to increase epithelial cell yield and culturing efficiency.

Materials and Reagents

1. Petri-dish 92 x 16 mm (SARSTEDT, catalog number: 82.1473)
2. Disposable scalpels (Swann Morton, catalog number: 0501)

3. 15 ml CELLSTAR® tubes (Greiner Bio One, catalog number: 188261)
4. 50 ml CELLSTAR® tubes (Greiner Bio One, catalog number: 227270)
5. 7 ml Bijoux tubes (Greiner Bio One, catalog number: 189176)
6. FisherBrand™ Nylon mesh cell strainer, 40 µm (Thermo Fisher Scientific, Fisher Scientific, catalog number: 11587522)
7. 0.2 µm Minisart® NML syringe filter (Sartorius Stedim Biotech, catalog number: 16534-K)
8. 20 ml syringes (BD, catalog number: 300613)
9. 60 ml syringes (BD, catalog number: 309653)
10. Sterile pipette filter-tips 1,000 µl (Alpha Laboratories, catalog number: ZP1250S)
11. Sterile pipette filter-tips 100 µl (Alpha Laboratories, catalog number: ZP1200S)
12. Fisherbrand™ glass Pasteur pipettes (Thermo Fisher Scientific, Fisher Scientific, catalog number: 1156-6963)
13. MS columns (Miltenyi Biotec, catalog number: 130-042-201)
14. Wallach Endocell® disposable endometrial cell sampler (Wallach Surgical Decices, catalog number: 908014A)
15. Human endometrial biopsies (see step A)
16. Cell culture media
 - a. DMEM/F12 (1:1) with phenol red (Thermo Fisher Scientific, Gibco™, catalog number: 31330-038)
 - b. L-glutamine (Thermo Fisher Scientific, Gibco™, catalog number: 25030-081)
 - c. Antibiotic/antimycotic (Thermo Fisher Scientific, Gibco™, catalog number: 15240-062)
 - d. β-estradiol (Sigma-Aldrich, catalog number: E2758)
 - e. Recombinant human insulin (Sigma-Aldrich, catalog number: 91077C)
 - f. Acetic acid, glacial ≥ 99.7% (Sigma-Aldrich, catalog number: 695092)
17. Tissue digestion media
 - a. DMEM/F12, phenol-free media (Thermo Fisher Scientific, Gibco™, catalog number: 11039-021)
 - b. Collagenase from *Clostridium histolyticum* (Sigma-Aldrich, catalog number: C9891-500MG)
 - c. DNase I from bovine pancreas (Roche Diagnostics, catalog number: 11284932001)
18. Trypsin-EDTA, 0.25% (Thermo Fisher Scientific, Gibco™, catalog number: 25200-056)
19. Ficoll-paque plus medium (GE Healthcare, catalog number: 17-1440-02)
20. Separation buffer (see Recipes)
 - a. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A2153)
 - b. Phosphate-buffered saline (PBS) (Dulbecco A) Oxoid™ (Thermo Fisher Scientific, Thermo Scientific™, catalog number: BR0014G)
21. PE anti-human SUSD2, clone: W5C5 antibody (Biolegend, catalog number: 327406)
22. Anti-PE microbeads (Miltenyi Biotec, catalog number: 130-048-801)
23. Ethanol, absolute (Thermo Fisher Scientific, Fisher Scientific, catalog number: 10437341)
24. Sterile distilled water

25. Dextran-coated charcoal (DCC)-treated FBS (see Recipes)
 - a. Charcoal (Sigma-Aldrich, catalog number: C9157)
 - b. Dextran 70 (Sigma-Aldrich, catalog number: 1179741)
 - c. Fetal bovine serum (FBS) (Thermo Fisher Scientific, Gibco™, catalog number: 10500-064)
26. Digestion media (see Recipes)
27. Culture media (see Recipes)

Equipment

1. 25 cm² CELLSTAR® culture flasks (Greiner Bio One, catalog number: 690175)
2. 75 cm² CELLSTAR® culture flasks (Greiner Bio One, catalog number: 658175)
3. 5 ml serological pipettes (Greiner Bio One, catalog number: 606180)
4. 10 ml serological pipettes (Greiner Bio One, catalog number: 607180)
5. Pipette controller/pipette aid (e.g., STARLABS, catalog number: S7166-0010)
6. Vacuum-driven 0.22 µm filtration system (EMD Millipore, catalog number: SCGPT05RE)
7. LUNA™ BF automated cell counter (Logos Biosystems, catalog number: L10001)
8. LUNA™ cell counting slides (Logos Biosystems, catalog number: L12001)
9. miniMACS separator (Miltenyi Biotec, catalog number: 130-042-102)
10. MACS multistand (Miltenyi Biotec, catalog number: 130-042-303)
11. Walker Class II cell culture microbiological safety cabinet (Walkers Safety Cabinets, model: Class II MSC)
12. Fisherbrand™ FB 70155 aspirator (Thermo Fisher Scientific, Fisher Scientific, catalog number: 11533485)
13. Grant Instruments water bath (Grant Instruments, model: OLS200)
14. Thermo Scientific Heracell™ 150i humidified tissue culture incubator (set at 37 °C and 5% CO₂) (Thermo Fisher Scientific, catalog number: 51026280)
15. Sigma 3-16KL bench-top centrifuge (Sigma Laborzentrifugen, model: 3-16KL)
16. Bright-field Leica DMIL microscope (Leica Microsystems, model: Leica DMIL)

Procedure

A. Collection of human endometrial biopsies

Endometrial biopsies are obtained from women attending the Implantation Clinic, a dedicated research clinic at University Hospitals Coventry and Warwickshire National Health Service Trust. All research was undertaken with full ethical approval and with written informed consent obtained from all participants in accordance with the guidelines in The Declaration of Helsinki 2000. Biopsies are taken during the secretory phase of the menstrual cycle using an Endocell cannula, starting from the uterine fundus and moving downward to the internal cervical ostium. The endometrial biopsy is placed in a labelled Bijoux tube containing 5 ml cell culture media, and processed immediately.

Notes:

- a. *Biopsy sizes vary considerably depending on the patient and clinical professional performing the procedure. The hormonal status of the patient and the stage of menstrual cycle in which biopsies are obtained will effect yielding and cell composition due to the transient phasic dynamics of the endometrium. Our biopsies are timed to the secretory phase of the menstrual cycle, but readers are encouraged to time collections around their own experimental hypothesis.*
- b. *Typically, biopsies have a uniform thickness (see Figure 1B) but can vary in length from < 1-6 cm. Tissue is also fragile and may fragment on collection or in transit, or be contaminated with blood or mucus. Readers should follow the protocol exactly in all situations. The only exception is when mucus content exceeds that of endometrial tissue. In this case the biopsy is discarded.*

B. Tissue digestion, isolation and culture of Human Endometrial Stromal Cells (HESCs)

Note: Ensure sterility. Work in a Class II microbiological safety cabinet and ensure full aseptic technique.

1. Pre-prepare digestion media and pre-warm to 37 °C in a water bath.
2. Decant as much media as possible from the Bijoux vial into the lid of the Petri-dish without discarding tissue. Transfer biopsy and any remaining media into the Petri-dish (Figure 1A).

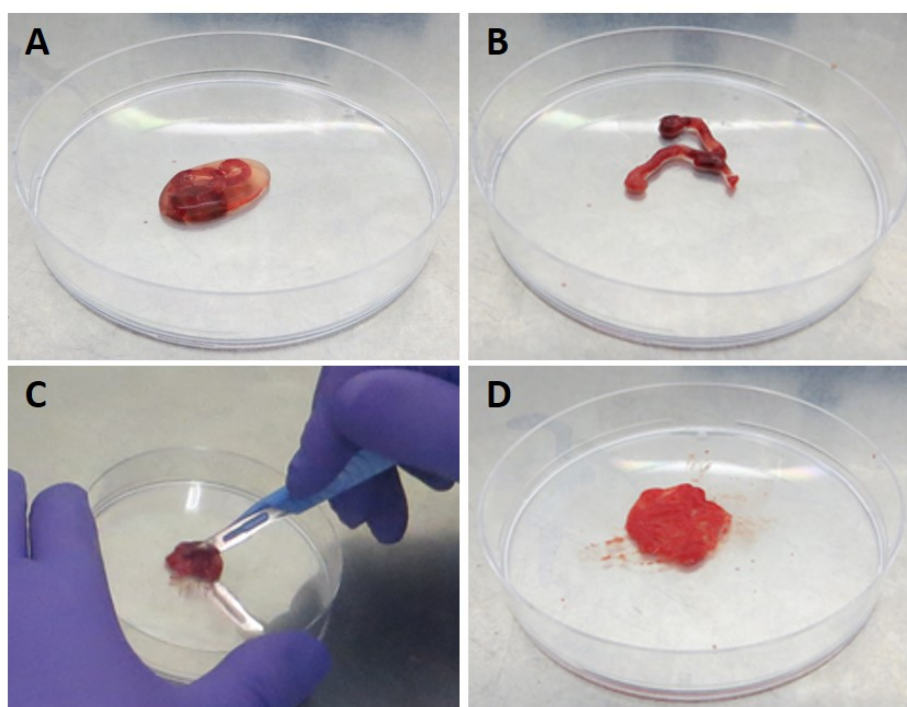


Figure 1. Preparation of endometrial biopsies for digestion. Collect whole biopsies in culture media and process immediately (A). Remove all media using a manual pipette (B) before dicing using a down-ward tapping motion with a sterile scalpel (C) for 5 min or until pulp-like (D). Tissue pieces are then digested for 1 h at 37 °C with collagenase and DNase I.

3. Manually aspirate the excess media from around the tissue using a P1000 filter tip (Figure 1B).
Note: Do not use an aspirator or risk losing tissue.
4. Dice the tissue with the scalpel using a downward tapping motion for at least 5 min (Figure 1C) or until large pieces have disappeared and the tissue appears pulp-like (Figure 1D).
5. Add 10 ml of digestion media to the Petri-dish through a 20 ml syringe and 0.2 μ m syringe filter.
6. Transfer the media with the tissue fragments to a 50 ml conical tube using a 10 ml serological pipette.
7. Shake the tube for 15 sec and incubate at 37 °C for 1 h. Shake for 15 sec at 20 min intervals to aid digestion.
8. After 1 h add 10 ml of culture media to neutralize enzymatic activity and centrifuge the tube at 280 x g for 5 min at room temperature.
Note: It is at this stage that epithelial cells (HEECs) (see step C) or perivascular stem-cells (see step D) can be isolated for separate cultures.
9. After centrifugation, aspirate the supernatant, re-suspend the cell pellet in 15 ml of culture media and transfer to a 75 cm² culture flask.
Note: Tilt the flask gently to distribute the cell suspension evenly across the culture surface. Avoid rotational mixing to risk concentrating cells in the center.
10. Incubate at 37 °C in a humidified 5% CO₂ environment.
11. Change the culture media after 6-18 h to remove blood cells, tissue debris and any unattached human endometrial epithelial cells (HEECs). Examination under the microscope should reveal sub-confluent stromal cells (Figure 2A).
Note: Confluency will vary depending on size and quality of biopsy. At this stage (see also Figure 6), immune cells can be collected from the supernatant and separated by antigen-specific magnetic separation or FACS, the details of which go beyond the remit of this protocol but readers are directed elsewhere (e.g., Manaster et al., 2008; Basu et al., 2009).
12. Cells will continue to proliferate (Figure 2B) until confluent (Figure 2C). Change the culture media every other day until passage (see step E) or assay.
Notes:
 - a. HEEC contamination (Figure 2D) should be minimal, please refer to 'Notes' section.
 - b. Typical HESC yields vary considerably depending on digestion efficiency, mucus and blood content and varying cell attachment rates, but they will correlate with the size and quality of the tissue. As a guide we would expect to obtain $1-5 \times 10^5$ cells from a small biopsy (< 1 cm), $0.5-1 \times 10^6$ for an average biopsy (2-3 cm) and $1-2 \times 10^6$ for larger biopsies (> 4 cm) (counted 24 h after seeding). Readers are encouraged to allow cells to proliferate in culture until desired cell numbers are obtained.

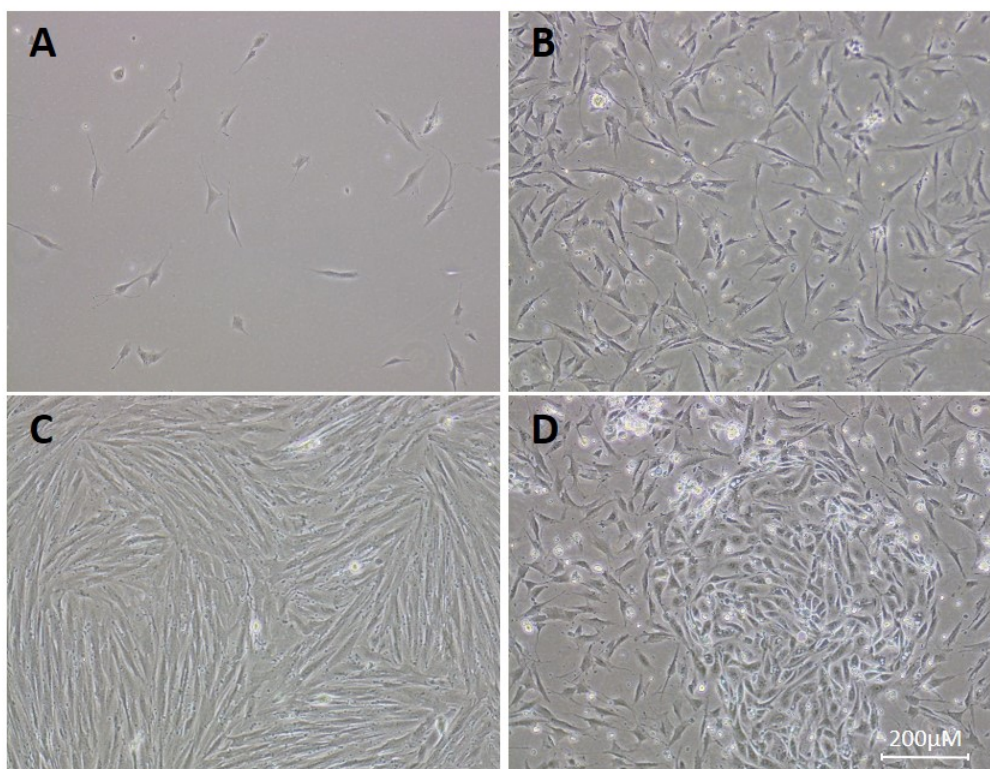


Figure 2. Cultured HESCs (bright-field microscope). A. HESC culture day one post-seeding. B. HESC culture day three post-seeding. C. Confluent culture of HESCs ready for passage or seeding. D. Culture of HESCs contaminated by HEECs, present as a whorl-like group of cells in the center.

C. Separation of HEECs

1. After digestion (step B8, refer also to Figure 6), filter the cell solution through a 40 μ m nylon mesh cell-strainer. Flow-through will contain stromal, red blood and immune cells, but endometrial gland clumps are retained in the strainer (Figure 3A).
2. Back-wash the filter using 20 ml of additive-free DMEM/F12 media and collect the glandular clumps in a 50 ml tube (Figure 3B).
3. Centrifuge at 280 \times g for 5 min, room temperature.
4. Aspirate the supernatant and re-suspend the pellet in 1 ml 0.25% trypsin-EDTA to dissociate any clumps. Glandular clumps do not attach well to substrate and can be lost during media changes. This additional trypsin-dissociation step differs from previous protocols (Chen and Roan, 2015), and increases HEEC yield by dispersing clustered cells.
5. Incubate the tube at 37 $^{\circ}$ C for 10 min.
6. Add 9 ml of culture media and dissociate glandular clumps by vigorous pipetting up and down. A single cell solution of HEECs should result.
7. Centrifuge at 280 \times g for 5 min, room temperature.
8. Aspirate the supernatant and re-suspend and seed the HEEC as required.

Notes:

- a. *HEEC yielding will depend on biopsy size and quality. Freshly isolated HEECs counted after step C8 typically yield $1-3 \times 10^5$ from small biopsies (< 1 cm), $3-9 \times 10^5$ for medium (2-3 cm) and 1×10^6 for large biopsies (> 4 cm). However, readers are encouraged to allow proliferation in culture to obtain desired numbers.*
- b. *The culture of HEECs requires non-standard culture techniques that go beyond the remit of this protocol. Readers are therefore directed elsewhere (e.g., Chan et al., 2004; Defrere et al., 2005; MacDonald et al., 2007).*

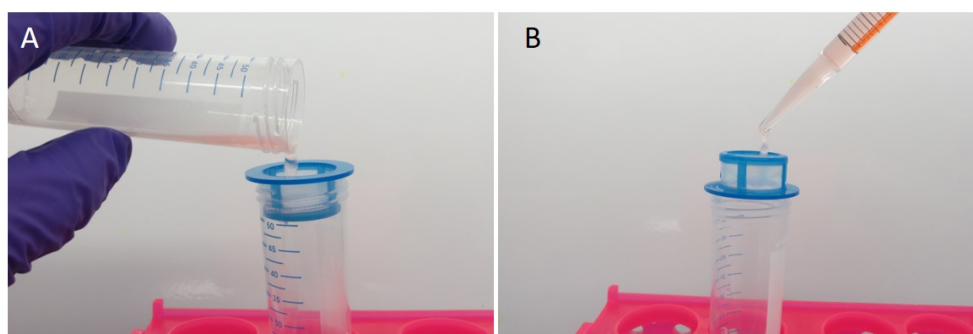


Figure 3. Separation and isolation of HESCs and HEECs. A. Following tissue digestion, collect HESCs as flow-through through a 40 μ m cell strainer. B. Collect glandular epithelial clumps by back-washing (note inverted cell strainer) and disperse into single cell HEECs suspensions via trypsin incubation.

- D. Isolation of endometrial perivascular progenitor mesenchymal stem-like cells
 1. Following step B8 (refer also to Figure 6), aspirate the supernatant and re-suspend the pellet in 8 ml culture media.
 2. Underlay 4 ml of Ficoll-paque to the bottom of the tube (Figure 4A). Ficoll-paque is a density gradient media used to separate out red blood cells.
Note: It is important to have two distinct layers before centrifugation (Figure 4B). To underlay Ficoll-paque, fill a 5 ml serological pipette, but dispense only 4 ml to the bottom of the tube, thus avoiding expulsion of bubbles and air mixing. Discard the remaining 1 ml. Dispense the 4 ml slowly and turn down the speed on the pipette-aid to its slowest setting. Avoid mixing at all times by handling carefully. Do not mix, knock or invert.
 3. Centrifuge at 770 x g for 10 min, room temperature. Red blood cells will pellet and HESCs will reside within the interphase between the 2 layers (Figures 4C and 4D).

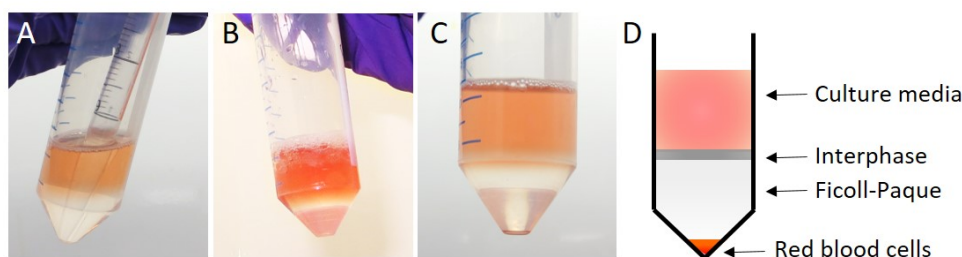


Figure 4. Separation of red blood cells using Ficoll-paque. A. Slowly underlay 4 ml Ficoll-paque beneath the digested tissue. B. 2 distinct layers should be visible. C-D. After centrifugation, red blood cells are pelleted and HESCs and HEECs as well as immune cells remain in the interphase from where they can be collected.

4. Carefully aspirate the majority of supernatant and collect the interphase containing the stromal cells and transfer to a 15 ml tube.
5. Add 8 ml of culture media and mix well with a pipette.
6. Wash cells free of Ficoll-paque by centrifugation ($280 \times g$ for 5 min, room temperature), aspiration of supernatant and resuspension in 10 ml culture media.
7. Repeat step D6 twice more.
8. Aspirate the supernatant and re-suspend the cell pellet in 5 ml of culture media and count the cells using an automated cell counter or haemocytometer.
9. Centrifuge at $280 \times g$ for 5 min, room temperature.
10. Aspirate the supernatant and re-suspend the cell pellet in separation buffer (see Recipes) containing PE-conjugated W5C5 antibody. Use 100 μ l of separation buffer and 5 μ l of antibody per 10^6 cells.
11. Mix well and incubate for 20 min in the dark at 4 °C.
12. Wash the cells to remove unbound antibodies by adding 1 ml of separation buffer per 10^6 cells and centrifuge at $280 \times g$ for 5 min at room temperature.
13. Aspirate the supernatant completely and re-suspend the cell pellet in separation buffer containing anti-PE microbeads. Use 80 μ l of buffer and 20 μ l of anti-PE microbeads per 10^7 cells.
14. Mix well and incubate for 20 min in the dark at 4 °C.
15. Wash the cells by adding 1 ml of separation buffer per 10^6 cells and centrifuge at $280 \times g$ for 5 min at room temperature.
16. Aspirate the supernatant completely and re-suspend up to 10^7 cells in 500 μ l of separation buffer.
17. Place the MACS separator on the multi-stand and place the MS column in the MACS separator (Figure 5A).
18. Mix the cell suspension by pipette, and apply to the column. Avoid adding air bubbles to the column.

19. Collect unlabelled cells that pass through and wash MS column by addition of 500 μ l separation buffer three times. Only add fresh 500 μ l of buffer when the column reservoir is empty. Collect total effluent. This is the W5C5 negative fraction.
20. Remove the MS column from the separator and place it in a sterile 15 ml tube.
21. Immediately add 1 ml separation buffer into the column and flush out the magnetically labelled cells by firmly pushing the plunger into the column (Figure 5B). Flow-through will now contain the W5C5 positive fraction.
22. To increase purity of the magnetically labelled cells, repeat magnetic separation on positive fraction (steps D18 to D21) using a new MS column.
23. Centrifuge at 280 \times g for 5 min at room temperature.
24. Aspirate the supernatant, re-suspend the cell pellet and seed as required.

Although a high level of patient-to-patient variability is observed, isolated W5C5⁺ cells typically constitute between 4-8% of stromal cell populations (Murakami *et al.*, 2013). They maintain many mesenchymal stem cell characteristics (Masuda *et al.*, 2012), and can be cultured and differentiated (Ulrich *et al.*, 2014) and assessed for clonogenicity using CFU (Colony Forming Units) assay (Masuda *et al.*, 2012; Murakami *et al.*, 2013; 2014) as required.

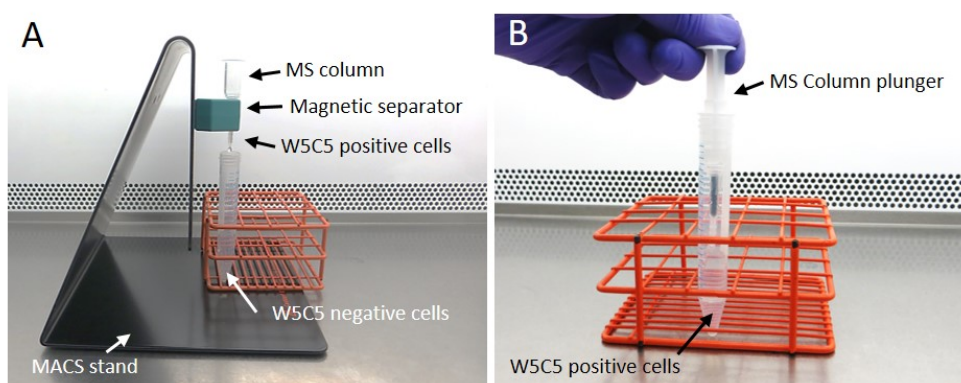


Figure 5. Magnetic separation of endometrial perivascular progenitor mesenchymal stem-like cells. Following antibody-labelling of W5C5⁺ cells (steps D9-D15), gravity-feed cell suspensions through columns in the magnetic stand. The flow-through will be the negatively labelled fraction (A). Remove the column from the magnetic stand and flush-through W5C5 positively labelled cells by immediate addition of 1 ml separation buffer and expulsion via the plunger (B). These steps can be repeated with the positive fraction using a fresh MS column to increase purity.

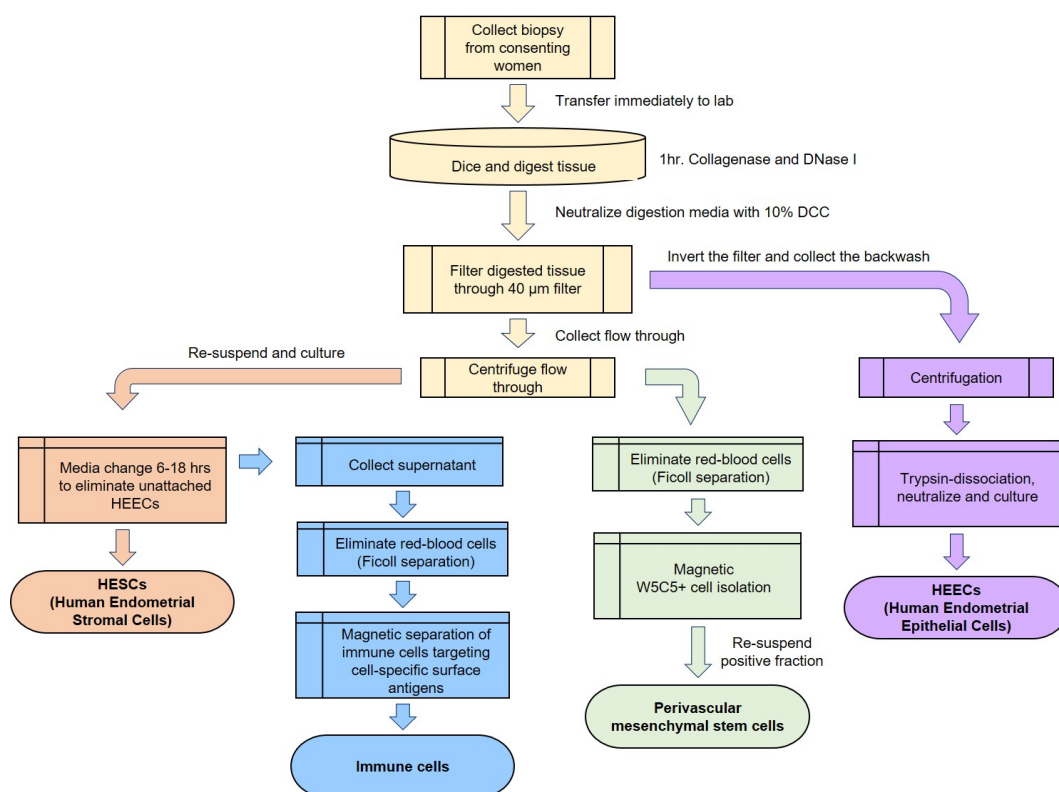


Figure 6. Work flow to separate different cell types within human endometrial biopsies

E. Passage of HESCs

1. Passage cells at 80-90% confluency (see Figure 2C).
2. Pre-warm culture media, sterile PBS and 0.25% trypsin-EDTA to 37 °C in the water bath.
3. Aspirate the culture media from the flask.
4. Add 10 ml of PBS, rinse the cell monolayer and aspirate.
5. Add 2 ml of 0.25% trypsin-EDTA solution, tilting the flask to ensure the solution covers the entire surface and return to incubator for 2-3 min or until cells have dislodged.
Note: Cells can be loosened by gentle agitation of the flask and checked under the light microscope.
6. Add 8 ml of culture medium into the flask to neutralize the trypsin and pipette the media repeatedly over the bottom of flask to wash any remaining cells.
7. Transfer the cell suspension to a 15 ml tube and centrifuge for 5 min at 280 x g, room temperature.
8. Re-suspend pellet in 9 ml culture media and seed 3 ml into a new 75 cm² culture flask containing 12 ml culture media. For maintenance of culture, cells are usually split at a ratio of 1:3, but can at this stage be seeded into plasticware suitable for desired experiments.

Notes

1. When preparing DCC-FBS the vacuum filtration stops

- a. Filtration units are easily clogged. Avoid aspirating the charcoal from the bottom of the tube.
 - b. Change the filtration system. Sometimes it takes two filter changes to accomplish total filtration.
2. Culture is contaminated with epithelial cells (see Figure 2D)
 - a. After biopsy digestion, (step B8) filter the solution using a 40 μ m cell strainer. The stromal cells pass through the filter and the majority of glandular clumps are retained.
 - b. Perform the first media change earlier (3 to 6 h). Most stromal cells, but few epithelial cells, will be attached at this time.
 - c. During the passage of the contaminated flask reduce the trypsin time to 2 min. Epithelial cells take longer to detach.
3. Few HESCs have attached and they are slow growing
 - a. If the biopsy is too small seed the cells in a 25 cm² culture flask instead of 75 cm².
 - b. Check for infection. The presence of a bacterial or fungal infection would restrict cell growth by starving cells of nutrients. Infections manifest in different forms but readers should be concerned by cloudy media, fungal colonies, or bacterial or fungal spores viewed under a light microscope. If infection is suspected, discard culture and bleach cells and media to avoid repeat infections.
4. The number of cells is too low even when the biopsy was large
 - a. This may be due to inadequate digestion. Ensure tissue slicing/chopping removes all large pieces to aid digestion.
 - b. Cell loss is possible during the Ficoll-paque separation. After the centrifugation with Ficoll-paque do not aspirate the top media phase. Transfer all media along with the interphase. Be sure to transfer the whole interphase even if you transfer a certain amount of Ficoll-paque along with it. The transferred Ficoll-paque will be cleared through washing.

Recipes

Note: Ensure sterility. Work in a Class II microbiological safety cabinet and ensure full aseptic technique.

1. Digestion media
 - 10 ml phenol and additive-free DMEM/F12 culture media
 - 0.5 mg/ml collagenase (prepare 50 mg/ml 100 μ l stock aliquots)
 - 0.1 mg/ml DNase I (prepare 10 mg/ml 100 μ l stock aliquots)
2. Dextran-coated charcoal (DCC)-treated FBS
 - a. Add 1.25 g of charcoal and 125 mg of dextran 70 to 500 ml of FBS, mix thoroughly and incubate at 56 °C in the water bath for 2 h, shaking every 30 min
 - b. Transfer the FBS to 50 ml tubes and centrifuge at 1,800 x g, for 30 min
 - c. Sterile-filter the supernatant using the vacuum-driven filtration system
 - d. Aliquot to 50 ml volumes, label and store at -20 °C

3. Culture medium
 - 500 ml DMEM/F-12 with phenol red
 - 50 ml (10% [v/v]) DCC-FBS
 - 5 ml (1% [v/v]) antibiotics/antimycotics
 - 5 ml (1% [v/v]) L-glutamine
 - 1 nM β -estradiol (prepare 100 μ M stock in ethanol, store at -20 °C, add 5 μ l)
 - 2 μ g/ml recombinant human insulin (prepare 10 mg/ml stock solution in acidified water [1.5%, v/v, acetic acid in sterile water, add 100 μ l])
4. Separation buffer (for magnetic separation of perivascular endometrial mesenchymal stem-like cells [0.5% BSA in PBS])
 - Dissolve 500 mg of BSA in 100 ml of sterile 1x PBS
 - Sterile-filter through 0.2 μ m filter
 - Store for up to a month at 4 °C before use

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