The Role of the Glucocorticoid Receptor Signalling Pathway within the Human Endometrium

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Summary:

The activation of the stress-responsive biological system, known as the hypothalamic-pituitary-adrenal axis leads to an increase in the synthesis of the glucocorticoids (GCs). The GCs regulate a number of diverse biological processes within the body by altering the transcription of an array of steroid responsive genes, mediated by the glucocorticoid receptor (GR). Along with the well-characterised sex steroid hormones, the human endometrium is also targeted by GCs due to the presence of GR within the healthy human endometrium Nevertheless, research is lacking in elucidating the role of stress and hence the GR signalling pathway on reproductive functions and fertility.

Objectives:

The principal aims were to present novel evidence for the expression of GCs and its nuclear receptor; GR in the endometrium of fertile and infertile patients and to relate these observations to key features of common reproductive endocrinological pathologies namely PCOS, endometriosis and unexplained infertility (UI). Secondary to this, was to establish the effect of GR signalling pathway activation in the process of endometrial decidual transformation in fertile and infertile patients.

Methodology:

This project employed the use of immunohistochemistry (IHC), cell culture, Real-time polymerase chain reaction (qPCR), immunoblotting, enzyme linked immunosorbant assays (ELISAs), chromatin immunoprecipitation (ChIP), cell morphology analysis and TRANSFAC promoter analysis.

Results:

Circulating cortisol levels and endometrial GR expression follow a distinct expression pattern throughout the normal menstrual cycle, with the lowest levels being observed during the secretory phase. Conversely, infertile patients exhibited significantly higher levels of both. In vitro analysis revealed that activation of the GR signalling pathway, although crucial for reproductive processes in the fertile patients, can result in delayed decidualization in the presence of the stress hormone cortisol. The effect of stress on the decidual response was able to be inhibited when a GR antagonist was used. ChIP analysis confirmed the presence of GR on the promoter regions of target genes in the presence of GCs during decidual transformation. An already hindered decidual response present in infertile patients was further exacerbated in the presence of the stress hormone. Moreover, a genetic switch from GR to mineralocorticoid receptor (MR) dominance, crucial for successful decidualization is inhibited in the infertile patients. Finally, a degree of crosstalk between GR and the sex steroid hormone signalling pathways also takes place during endometrial decidual transformation.

Conclusion:

In conclusion, it is apparent that the GR signalling pathway plays a crucial role in reproduction in the absence of the stress response. However, endometrial GR expression and circulating levels of the stress hormone cortisol is altered in infertile patients diagnosed with PCOS, endometriosis and UI. The activation of the GR signalling pathway due to the presence of the stress hormone cortisol is detrimental for crucial reproductive functions, including decidualization in both fertile and infertile patients.

Declaration/Statements

DECLARATION

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed ..... (candidate)

Date .....

STATEMENT 1

This thesis is the result of my own investigations, except where otherwise stated.
Other sources are acknowledged by footnotes giving explicit references.
A bibliography is appended.

Signed ..... (candidate)

Date .....

STATEMENT 2

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loans **after expiry of a bar on access approved by the Swansea University.**

Signed ..... (candidate)

Date .....

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Acknowledgements

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Abbreviations

A	
ABC	Avidin-Biotin Peroxidase Complex
ABMUHB	Abertawe Bro Morgannwg University Health Board
ACTH	Adrenocorticotrophic Hormone
AF-1	Activation Function 1
AF-2	Activation Function 2
AKT	Serine/Threonine Protein Kinase
ANOVA	Analysis of Variance Statistical Model
anPCOS	Anovulatory PCOS
AP-1	Activator Protein 1
APS	Ammonium Persulphate
AR	Androgen Receptor
ART	Assisted Reproductive Technology
ASRM	American Society for Reproductive Medicine
ATCC	American Type Culture Collection
ATP	Adenosine Tri-Phosphate
AU	Arbitrary Units
B	
BAG1	Bcl-2-Associated Athanogene-1
BLAST	Basic Local Alignment Search Tool
BMI	Body Mass Index
bp	base pair
BRG1	Brahma-related gene-1
BSA	Bovine serum albumin
BSP-1	Bone-Sialoprotein-1
C	
CA-125	Cancer Antigen 125
Ca^{2+}	Calcium
$CaCl_2$	Calcium Chloride
cAMP	Adenosine 3', 5'-Cyclic Monophosphate
CAT	Computerized Axial Tomography
CBG	Corticosteroid-Binding Globulin
CBP	CREB-Binding Protein
CBT	Cognitive Behavioural Therapy
CD44	Cell Differentiation Antigen
cDNA	Complementary DNA
C/EBP	CCAAT-enhancer-binding proteins
ChIP	Chromatin Immunoprecipitation
cm^2	Square Centimetre
CO_2	Carbon dioxide
CRE	Cyclic AMP Response Element
CRE	cAMP Regulatory Element
CREB	cAMP Response Element Binding Protein (also CBP)
CRH	Corticotropin-Releasing Hormone
CSF-1	Colony Stimulating Factor -1
C_t	Cycle Threshold
CVD	Cardiovascular Disease
D	
d	Days
DAPI	4', 6-diamidino-2-phenylindole
DBD	DNA Binding Domain
dH_2O	Deionized Water
DHEA	Dehydroepiandrosterone

DHT	Dihydrotestosterone
DMEM/F-12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNase1	Deoxyribonuclease 1
dPRL	Decidual Prolactin
DRIP	Vitamin D Receptor-Interacting Protein
E	
E	Cortisone
E_2	Estradiol
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked Immunosorbent Assay
ER	Estrogen Receptor
ESCs	Endometrial Stromal Cells
ESHRE	European Society of Human Reproduction and Embryology
F	
F	Cortisol
FBS	Foetal Bovine Serum
FFPE	Formalin Fixed Paraffin Embedded
FKBP	FK506 Binding Protein
FSH	Follicle-Stimulating Hormone
FSHR	Follicle-Stimulating Hormone Receptor
G	
g	gram
GC	Glucocorticoid
GnRH	Gonadotropin-releasing Hormone
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Elements
H	
h	Hours
H_2O	Water
HCG	Human Chorionic Gonadotropin
H&E	Haematoxylin and Eosin
HESC	Human Endometrial Stromal Cell Line
HMB	Heavy Menstrual Bleeding
HPA	Hypothalamic Pituitary Adrenal Axis
HPG	Human Pituitary Gonadotropin
HRP	Horseradish Peroxidase
HRT	Hormone Replacement Therapy
HSG	Hysterosalpingogram
HSP	Heat shock protein
I	
ICI	Intracervical Insemination
ICSI	Intracytoplasmic sperm injection
IGFBP-1	Insulin-like Growth Factor-binding Protein 1
IgG	Immunoglobulin
IHC	Immunohistochemistry
IVF	In vitro fertilization
IVF-ET	In vitro Fertilization and Embryo Transfer
J	
JAK	Janus kinase
K	
kDa	Kilodalton

KO	Knock-out
L	
L	Litres
LBD	Ligand Binding domain
LH	Luteinizing Hormone
LHR	Luteinizing Hormone Receptor
M	
M	Molar
MAPK	Mitogen-Activated Protein Kinase
Mg	Milligrams
Min	Minutes
MMPs	Matrix Metalloproteinase
MPA	Medroxyprogesterone Acetate
MR	Mineralocorticoid Receptor
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
N	
NAD+	Nicotinamide Adenine Dinucleotide (oxidised form)
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of Activated B cells
nGRE	Negative Glucocorticoid Receptor Response Elements
NICE	National Institute for Health and Care Excellence
NK	Natural Killer
NLS	Nuclear Localisation Signal
nm	Nanometre
nM	Nanomolar
NP-40	Nonyl Phenoxypolyethoxylethanol
NTD	N-terminal Transactivation Domain
O	
O_2	Oxygen
OC	Ovarian Cancer
OCP	Oral Contraceptive Pill
ovPCOS	Ovulatory PCOS
P	
P_4	Progesterone
PBS	Phosphate Buffered Saline
PCAF	P300/CBP-Associated Factor
PCOS	Polycystic Ovary Syndrome
PCR	Polymerase Chain Reaction
PCRH-RE	POMC- CRH responsive elements
PFA	Paraformaldehyde
PGC1	PPARγ coactivator-1
PGE_2	Prostaglandin E2
PID	Pelvic Inflammatory Disease
PKA	Protein Kinase A
POMC	Proopiomelanocortin
PR	Progesterone Receptor
PRL	Prolactin
PTSD	Post-Traumatic Stress Disorder
PVN	Paraventricular Nucleus
PVDF	Polyvinylidene difluoride
Q	
qPCR	Real-time PCR or quantitative PCR
R	
RA	Rheumatoid Arthritis

RBGO	Reproductive Biology and Gynaecological Oncology
RIPA	Radioimmunoprecipitation assay (buffer)
RNA	Ribonucleic Acid
RPM	Revolutions Per Minute
RPL19	Ribosomal Protein L19
S	
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SHG	Sonohysterogram
siRNA	Small Interfering RNA
SMRT	Silencing Mediator for Retinoid and Thyroid Hormone Receptors
SNS	Sympathetic Nervous System
SRC-1	Steroid Receptor Coactivator-1
STAT	Signal Transducer and Activator of Transcription
SUMO	Small Ubiquitin-like Modifier
T	
TBS	Tris Buffered Saline
TBS-T	TBS-Tween
TEMED	N, N, N', N'-Tetramethylethylenediamine
TF	Transcription Factor
TGS	Tris-glycine-SDS
Tm	Melt temperature
TRAP	Thyroid Hormone Receptor-Associated Protein
Tris	Trishydroxymethylaminomethane
TSG101	Tumour susceptibility gene 101
U	
U	Units
UI	Unexplained Infertility
uNK	Uterine Natural Killer cells
V	
v/v	Volume/Volume
W	
WB	Western Blot
WHO	World Health Organisation
Wt	Wild type
WT1	Wilms Tumour 1
w/v	Weight/Volume
Symbols	
^{o}C	Degrees Celsius
µg	Microgram
µL	Microlitre
µM	Micromolar
%	Percentage
Numbers	
11β-HSD1	11β-hydroxysteroid dehydrogenase type 1
11β-HSD2	11β-hydroxysteroid dehydrogenase type 2

CHAPTER 1

Introduction

1. Introduction

1.1 Stress

The world is full of stressors which have a significant impact upon our mood, mental well-being and behaviour and ultimately our health. These stressors have the ability to disrupt homeostasis within the body and our survival depends on our ability to generate appropriate stress responses. Acute stress responses in young and healthy individuals do not normally have a negative impact on our health and may also be adaptive. These adaptations include enhanced cognition, physical strength and analgesia, along with the inhibition of non-essential functions within the body such as reproduction and growth (Rogers and Lucchesi, 2014). Acute stressors generate responses which improve the chances of immediate survival at the expense of other processes within the body, where multiple stress signalling systems co-ordinate these adaptive responses. (Schneiderman et al, 2005).

On the other hand, chronic stress and continuous exposure to stressors can have significant long term effects on the human body and result in detrimental health impacts. It has been proposed that the relationship between stress exposure and disease is affected by various factors, such as the persistence of the stressors, the individual being exposed to the stress, the nature of the stressors and the individual's coping mechanisms (Schneiderman et al, 2005). Therefore stress-management may influence the development and progression of certain diseases.

1.1.1 Glucocorticoids

Glucocorticoids (GCs) are a group of steroid hormones which regulate a number of diverse biological processes within the body, such as the inflammatory and immune responses, cellular proliferation, apoptosis, foetal organ development, cardiovascular regulation and glucose metabolism amongst many others (Kadmiel and Cidlowski, 2013; Macfarlane, Forbes and Walker, 2008; Schmidt et al., 2004; Vilasco et al., 2011; Walker, 2007; Whirledge and Cidlowski, 2013). GCs also have potent anti-inflammatory and immunosuppressive effects within the body, which are being extensively studied (Marques, Silverman and Sternberg, 2009).

GCs exert their effects by altering the transcription of an array of steroid responsive genes mediated by its cognate receptor – the Glucocorticoid Receptor (GR) (Oakley and Cidlowski, 2013).

The clinical use of GCs dates back to the late 1940s when Philip Hench successfully treated the symptoms of rheumatoid arthritis with cortisone, for which he later received a Nobel Prize (Burns, 2016). Since then, GCs have transformed the field of medicine, where synthetic GCs are now being prescribed for several chronic inflammatory conditions, including asthma, skin and ocular infections and are also being used for immunosuppression in patients which are undergoing organs transplants. In addition to their anti-inflammatory properties, GCs have also been used for their anti-proliferative and anti-angiogenic actions in the treatment of cancers (Kadmiel and Cidlowski, 2013).

1.1.2 The Hypothalamic Pituitary Adrenal (HPA) Axis

The hypothalamic-pituitary-adrenal (HPA) axis is a stress responsive biological system which can be activated by inflammation, pain, infection and most importantly stress (Marques et al., 2009; Newton, 2000). Exposure to these stimuli or stressors leads to an increase in the production of the previously mentioned; GCs, which include the stress hormone cortisol, in a response mediated by the HPA axis (figure 1.1) (Newton, 2000).

A subset of paraventricular nucleus (PVN) neurons in the hypothalamus produce corticotropin releasing hormone (CRH) and following the exposure to stress, CRH is secreted into circulation where it consequently stimulates the pituitary to produce pro-opiomelanocortin (POMC) (Handa and Weiser, 2014). POMC is then cleaved to form adrenocorticotropic releasing hormone (ACTH) (Kadmiel and Cidlowski, 2013). ACTH enters the general circulation and stimulates the zona fasciculate of the adrenal cortex located in the adrenal gland resulting in the production of GCs (Armario et al, 2011). The production of GCs can inhibit the production of both ACTH and CRH production in an auto-regulatory negative feedback loop (Kadmiel and Cidlowski, 2013).

The HPA axis and the sympathetic nervous system (SNS) react to acute stressors by increasing heart rate, blood flow, oxygen flow and nutrient release and also by generating the fight or flight response (Wong et al, 2012). Additionally, the HPA axis also decreases reproductive, gastrointestinal and immune activities (Kadmiel and Cidlowski, 2013).

The synthesis and consequent release of the GCs is under dynamic circadian and ultradian rhythm, which is regulated ultimately by the hypothalamus (Spiga et al, 2014).

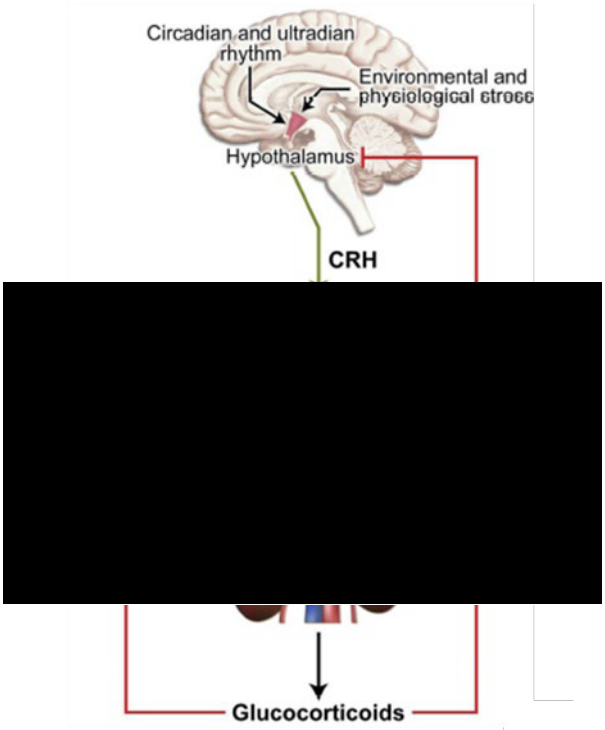


Figure 1.1. Diagram showing the hypothalamic-pituitary-adrenal axis which regulates glucocorticoid (GC) release within the human body. The synthesis and release of GCs is under dynamic circadian and ultradian regulation, where the corticotropin-releasing hormone (CRH) is secreted by the hypothalamus which results in the release of the adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. ACTH, in turn induces the synthesis and consequent secretion of cortisol from the adrenal glands into the bloodstream (Kadmiel and Cidlowski, 2013).

It is important to note, that not all stress responses are relayed through the PVN neurons of the hypothalamus which produce CRH. GCs exert a sustained secretory effect within the body, but the autonomic nervous system is able to respond to stressors within seconds. The autonomic nervous system is closely related with the sensory systems which monitor homeostatic disruptions within the body. For example, the brain stem is able to receive information about major homeostatic disruptions which would include pain, respiratory distress and blood loss (Ulrich-Lai and Herman, 2009). The autonomic pathways which are independent of hormonal changes are able to exert a stress response which results in behavioural changes. Therefore the autonomic nervous system and HPA axis are able to generate a stress response signalling pathway which can suppress many functions within the body, such as reproduction in response to homeostatic disruptions (Handa and Weiser, 2014).

1.1.2.1 Corticotropin Releasing Hormone (CRH) and Adrenocorticotropic Hormone (ACTH)

CRH regulates both basal and stress-induced HPA axis activity within the body (Vale et al, 1981). CRH is produced by the CRH-secreting neurones found within the brain, particularly the PVN (Armario, 2006). CRH is able to travel via portal circulation to the anterior pituitary gland, where it can bind to and activate its receptor; CRH type 1 receptor. To date, two CRH receptors have been identified in humans and other mammals, where the CRH type 1 receptor is expressed predominantly in anterior pituitary corticotrophin cells, whereas the type 2 receptor is more widely expressed in the brain and periphery (Aguilera et al, 2004). The activation of the CRH type 1 receptor, leads to the stimulation of expression and release of ACTH from the anterior pituitary gland. The expression of CRH is thought to be controlled by the recruitment of cAMP-response element binding protein (CREB) to a cAMP regulatory element (CRE) at approximately -200 bp in the proximal promoter of CRH (Seasholtz et al, 1988; Thompson et al, 1987).

ACTH is derived from its precursor; pro-opiomelanocortin (POMC), which has opioid, melanotropic and corticotropic activities (Bicknell, 2008). A CRH-induced rise in cAMP is responsible for the increase in POMC transcription and peptide synthesis within the anterior pituitary gland and also controls a rise in intracellular calcium levels, which leads to the secretion of ACTH (figure 1.2) (Jenks, 2009). CRH mediates the stimulation of POMC transcription via POMC- CRH responsive elements (PCRH-RE) (Jenks, 2009). A negative effect of GCs on POMC gene transcription is thought to be mediated by a GR complex binding to specific DNA sequences within the promoter region of POMC (Ratman et al, 2013). The release of ACTH from the anterior pituitary gland is mediated by signal transduction pathways via secondary messengers, such as protein kinase A (PKA), GCs, the JAK/STAT pathway (figure 1.2). Alterations in these pathways lead to changes in the expression of specific proteins and/or intracellular calcium levels, which control ACTH synthesis and release. ACTH in circulation is then able to bind to specific receptors in the adrenal gland (G-protein coupled type 2 melanocortin receptors), which leads to the biosynthesis of steroids (Armario, 2006).

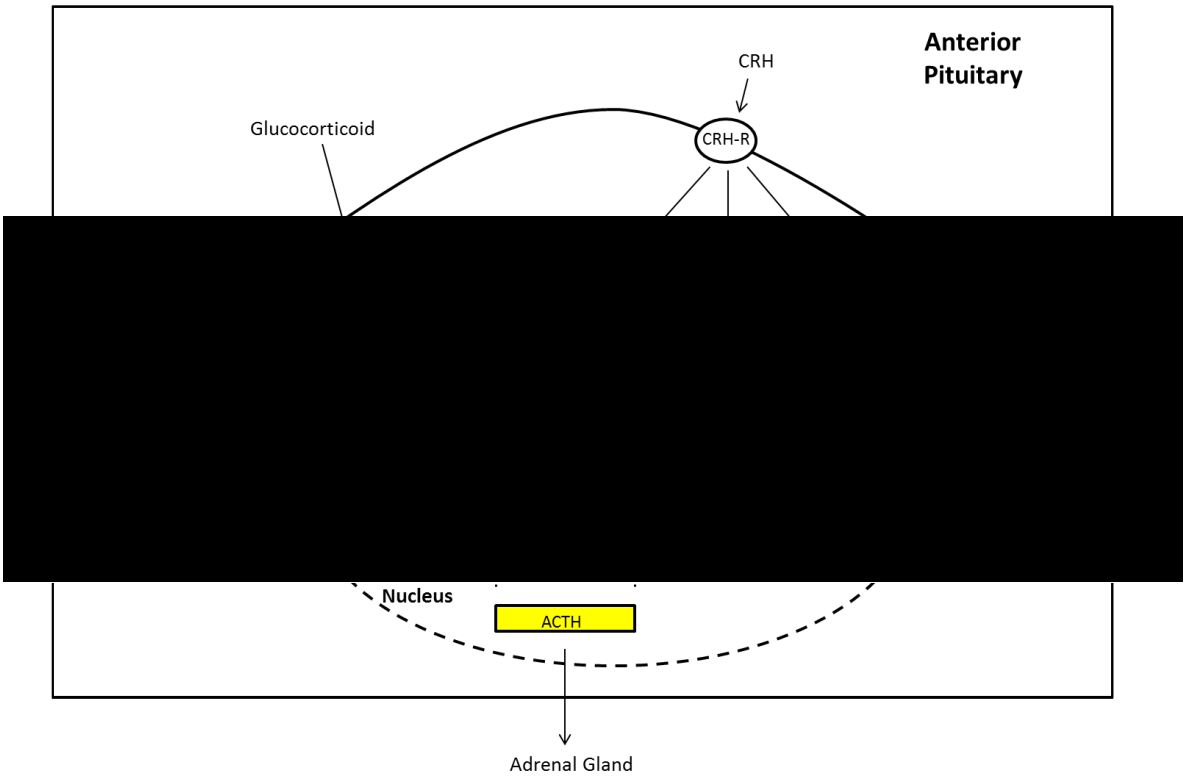


Figure 1.2 Diagram showing the intracellular signalling pathways regulating the transcription of the pro-opiomelanocortin (POMC) gene and the ultimate release of ACTH. Through its receptor, corticotropin-releasing hormone (CRH) induces cyclic monophosphate (cAMP) production, which activates protein kinase A (PKA) and the phosphorylation of the cAMP response element-binding protein (CREB) and induces POMC gene transcription. Additionally, CRH is also able to activate the mitogen-activated protein kinase (MAPK) pathways which also induce POMC gene transcription. Alternatively, glucocorticoids (GCs) acting through the GR pathway can repress the transcription of POMC. Once ACTH is synthesised, it can be released into circulation to the adrenal glands, leading to the biosynthesis of GCs (Adapted from: Stevens and White, 2010).

1.1.3 Cortisol

Cortisol (or compound F) is a 21-carbon glucocorticoid adrenal steroid hormone, which is produced by the zona fasiculata of the adrenal cortex (Kadmiel and Cidlowski, 2013). Cortisol is secreted in a diurnal pattern within the body, where it is at its highest concentration in the morning a few minutes after awakening. Cortisol levels continue to decrease during the day, where they reach their lowest concentration at approximately midnight. The diurnal secretion pattern of cortisol is very important, as it allows for the body to function at its maximum capacity and also allows for memory consolidation (Elder et al, 2014; Kadmiel and Cidlowski, 2013).

Glucocorticoids as the name suggests, play an important role in carbohydrate metabolism, where they are involved in the process of glyconeogenesis and gluconeogenesis (Kuo et al, 2015).

1.1.4 Cortisone

Cortisone (or compound E) is the biologically inactive form of the natural GCs within the human body (Kadmiel and Cidlowski, 2013). This adrenal steroid hormone was first discovered in 1935 by Edward Calvin who crystallised the compound from bovine adrenal glands (Hillier, 2007).

Cortisone has been used for many years within the clinic, in the 1940s – Phillip Hench and colleagues successfully treated the symptoms of rheumatoid arthritis (RA) with cortisone (Kadmiel and Cidlowski, 2013; Burns, 2016). Following this, cortisone was launched as a licensed pharmaceutical in 1950 and since then has remained among the most widely prescribed medications in the World. Despite its popularity as a medication, it has been found that with high dosage and long-term usage the potent effects of cortisone were countered by negative side effects such as excessive salt and water retention, increased gastric acidity and psychosis (Hillier, 2007). Therefore, considerable effort was directed at finding a cortisone derivative with decreased toxicity and improved efficacy (Feiser and Feiser, 1959).

Interestingly, even though it was determined that cortisone was an inactive molecule which required it to be metabolised to the active hormone cortisol in order for it to exert any effects, it is the pre-dominant steroid hormone in foetal tissues where levels start to decline immediately following birth (Nomura et al, 1996). This is due to the inactivation of the active hormone cortisol in the foetus which protects it from the inhibitory effects of cortisol, such as growth inhibition (Murphy, 1980).

1.1.5 Corticosteroid Binding Globulin

The bio-availability of natural, biologically active GCs such as cortisol, within the blood and tissues is regulated by corticosteroid binding globulin (CBG) and by the locally expressed 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes respectively, which will be explained in more detail in section 1.1.6 (Kadmiel and Cidlowski, 2013; Marques et al., 2009; Volden and Conzen, 2013).

In order for active GCs to exert their numerous effects within the body, unbound GCs need to diffuse across the cell membrane where they consequently bind cytosolic receptors and control the expression of target genes (Marques et al., 2009). However, it is known that approximately 90% of all circulating GCs are bound to CBG and are not available to diffuse across the cell membrane and alter gene transcription. Therefore, the relative concentrations of CBG present within the serum are important to help decipher the levels of biologically active GCs (Henley et al, 2016; Marques et al., 2009).

1.1.6 11β-Hydroxysteroid Dehydrogenase (11β-HSD) Enzymes

The 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes regulate the availability of active GCs within organs and tissue by catalysing the conversion of active GCs (cortisol) into the inactive form (cortisone), and vice versa (figure 1.3) (Marques et al., 2009).

There are two isoforms of 11β-HSD present within the body. The type 1 isoform; 11β-HSD type 1, acts as a reductase enzyme and converts the inactive GC; cortisone into the active GC; cortisol, using NADPH as a co-factor (Zhu et al., 2016). Whereas, the type 2 isoform; 11β-HSD type 2, acts as an oxidase/dehydrogenase enzyme and catalyses the reverse reaction, using NAD+ as a co-factor (Marques et al., 2009; Zhu et al., 2016).

Figure 1.3. Diagram showing the conversion of the inactive cortisone to the biologically active cortisol and vice versa. The unbound biologically active cortisol can be converted to the inactive form; cortisone by the 11β-hydroxysteroid dehydrogenase (11β-HSD) type 2 isoform. The 11β-HSD type 1 isoform catalyses the reverse reaction (Kadmiel and Cidlowski, 2013).

The systemic conversion of cortisone to cortisol is via hepatic 11β-HSD1 enzyme activity, whereas 11β-HSD2 is primarily expressed in the kidney (Hillier, 2007). Apart from the

systemic conversion, cortisol and cortisone are also freely interconverted in peripheral tissues where changes in their dynamic equilibrium may regulate GC activity in various tissues (Nomura et al, 1996). In addition to the liver and kidney, the 11β-HSD enzymes are also expressed within adipose tissue, skeletal muscle, vasculature and heart, bones, lung and within the female reproductive system where both the ovary and uterus express both 11β-HSD1 and 11β-HSD2 (Chapman, Holmes and Secki, 2013).

1.2 Glucocorticoid Receptor (GR)

Glucocorticoids (GCs) are the primary output of the HPA axis and the actions of these steroid hormones are exerted through the glucocorticoid receptor (GR). GR is a member of the nuclear hormone receptor family of transcription factors (TFs) which are ubiquitously expressed throughout the body and is encoded by the gene; NR3C1 (Newton, 2000; Schlossmacher, Stevens, and White, 2011; Walker, 2007). GR is related to the mineralocorticoid, androgen, estrogen, progesterone, vitamin D and retinoic acid steroid hormone receptors. There is known to be high levels of conservation amongst these receptors in regards to their sequence and structure (Revollo and Cidlowski, 2009).

The rat GR was the first steroid hormone receptor to be successfully cloned, which allowed for the analysis of corticosteroid hormone action (Stewart and Mason, 1995).

1.2.1 Isoforms of the Glucocorticoid Receptor

GR is encoded by the nuclear receptor subfamily 3, group C, number 1 (NR3C1) gene which is located on chromosome 5 in humans (Marques et al, 2009). The human NR3C1 gene contains nine exons with the protein coding region formed by exons 2-9 (Kadmiel and Cidlowski, 2013). The transcription of GR is regulated by the 5' untranslated region (exon 1) and the numerous first exons present in the gene are thought to be involved in regulating and fine-tuning levels of GR within the body (Turner et al, 2010). NR3C1 also has a variable 3' region in exon 9, which encodes various splice variants, therefore alternative splicing and translational events leads to the formation and production of numerous GR isoforms. The GR isoforms and splice variants include: GRα, GRβ and the less studied GR-γ, GR-P and GR-A (Marques et al., 2009; Turner et al, 2010; Kadmiel and Cidlowski, 2013; Vilasco et al, 2011). GRα and GRβ are generated by two splice 3' exons; exons 9α and 9β respectively (figure 1.4). These isoforms are therefore identical in their amino acid sequence,

but differ in their C-termini, with GRα being the pre-dominant isoform. (Kadmiel and Cidlowski, 2013).

It is known that GRα mediates GC action within cells and controls the transcription of GR target genes, however the GRβ isoform is defective in ligand binding, and is thought to act as a dominant inhibitor of the primary isoform; GRα (Marques et al., 2009; Newton, 2000).

The GR isoforms can undergo key post-translational modifications, such as phosphorylation, acetylation, ubiquitination and SUMOylation. (Anbalagan et al, 2012; Oakley and Cidlowski, 2013). These modifications play a key role in the regulation of many cellular events and can alter the function of GR as a TF (Kadmiel and Cidlowski, 2013). GR is known to undergo phosphorylation modifications on specific residues on binding to biologically active GCs. This in turn determines the GR activity in the cells, as the activation or repression of GR mediated transcription is consequently determined by its phosphorylation state (Vilasco et al, 2011).

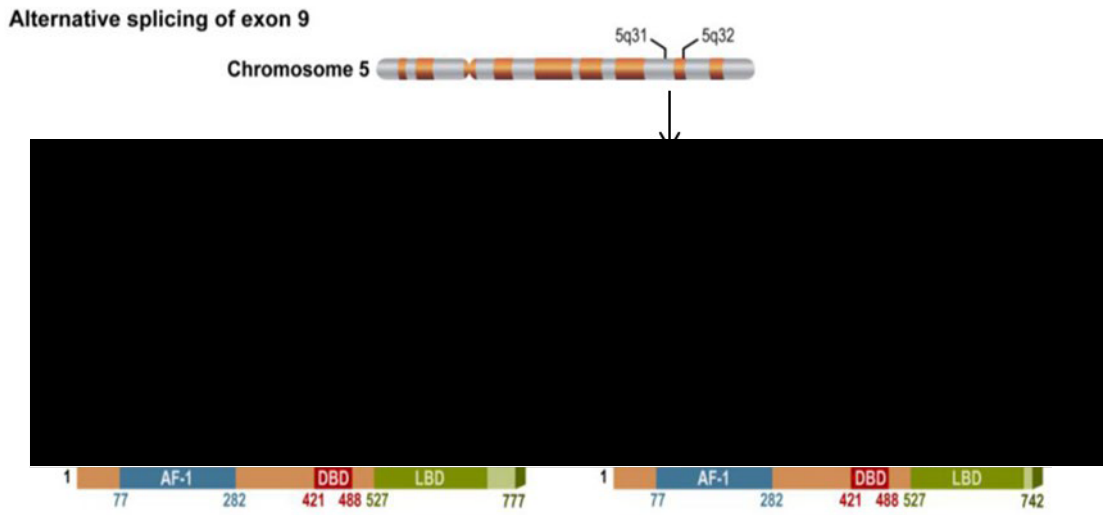


Figure 1.4. Diagram showing the alternative splicing events in the NR3C1 gene which yield the GR isoforms. GR undergoes alternative splicing to yield functionally distinct isoforms of GR. GR contains 9 exons with the protein coding region formed by exons 2-9. Exon 1 contains the 5'-untranslatd region of the gene. Alternative splicing of NR3C1 at exon 9 generates the predominant GRα and GRβ isoforms which differ at their C-terminus. hGR = human GR (Kadmiel and Cidlowski, 2013).

1.2.2 Structure of the Glucocorticoid Receptor

The pre-dominant GR isoform; GRα is a 777 amino acid protein, whereas GRβ is only a 742 amino acid protein and is expressed at much lower levels throughout the human body (Revollo and Cidlowski, 2009). The less studied GR-P lacks both exons 8 and 9 which

results in the translated protein having a truncated ligand binding domain – which is thought to enhance the activity of the pre-dominant isoform (Revollo and Cidlowski, 2009; Turner et al, 2010).

GRα and GRβ are both modular proteins containing an N-terminal transactivation domain (NTD), a central DNA-binding domain (DBD), a C-terminal ligand-binding domain (LBD) and also a flexible 'hinge region' separating the DBD and LBD (figure 1.5) (Kadmiel and Cidlowski, 2013).

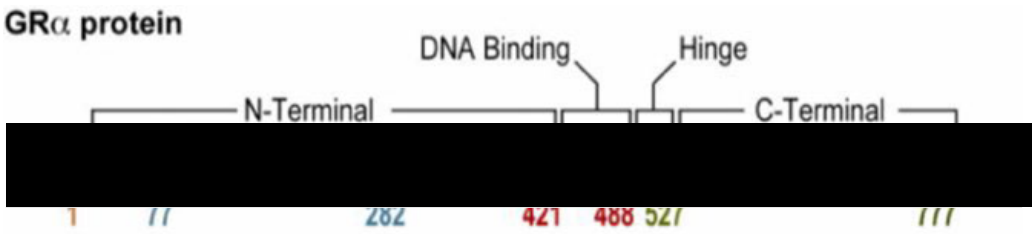


Figure 1.5. Diagram showing the protein structure of the glucocorticoid receptor (GR). GR is a modular protein containing an N-terminal transactivation domain (NTD) which comprises of a strong transcriptional activation function (AF-1), a central DNA-binding domain (DBD) and a C-terminal ligand-binding domain (Kadmiel and Cidlowski, 2013).

The NTD has a strong transcriptional activation function (AF-1), which allows for the recruitment of co-regulators and transcriptional machinery (Kadmiel and Cidlowski, 2013). GCs are able to bind the hydrophobic pocket of the LBD which is shown in figure 1.5 as the NLS or nuclear localisation signal, this binding results in the recruitment of co-regulators to the second activation function region (AF-2) also located within the C-terminal domain (Oakley and Cidlowski, 2013). The DBD, hinge region junction and the LBD, each contain a nuclear localisation signal which allows the translocation of the receptor to the nucleus (Oakley and Cidlowski, 2013).The DBD of GRα contains two zinc finger motifs which are able to intercalate with specific DNA sequences in the promoter region of target genes (Nicolaides et al, 2010; Freedman et al, 1988; Luisi et al, 1991). It is also the most conserved region of the protein amongst the other members of the nuclear hormone receptor superfamily (Kadmiel and Cidlowski, 2013).

1.2.3 Expression and Function of the Glucocorticoid Receptor and Glucocorticoids

GR is ubiquitously expressed throughout the body, although there are differences in expression between tissues. The effect of GCs in these different tissues is influenced by a

combination of factors, which include the expression of the diverse GR splice variants and co-regulator proteins and also the role of tissue-dependent post-translational modifications of GR (Schlossmacher et al, 2011).

Glucocorticoids and hence GR have many functions within the human body as seen in figure 1.6. For example, GCs play a role in the regulation of cellular proliferation and apoptosis, foetal organ development, cardiovascular regulation and glucose metabolism (Macfarlane et al, 2008; Schmidt et al, 2004; Vilasco et al, 2011; Walker, 2007; Whirledge and Cidlowski, 2013). GCs also play a significant role in the inflammatory and immune responses, where they have potent anti-inflammatory and immunosuppressive effects (Marques et al, 2013). Furthermore, due to these effects - GCs are the most widely used immunosuppressive and anti-inflammatory agents for the treatment of inflammatory disorders (Cvoro et al, 2011).

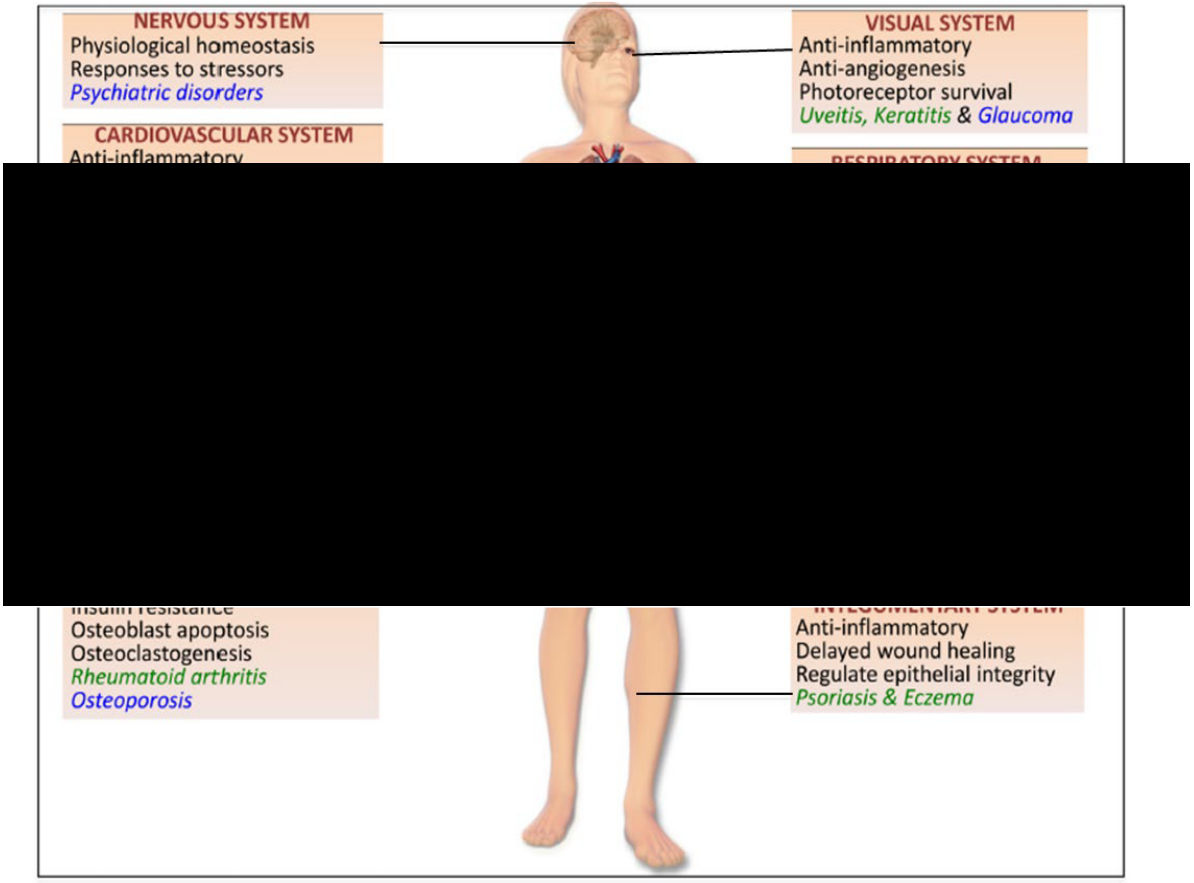


Figure 1.6. Diagram showing the role of glucocorticoids (GCs) and the glucocorticoid receptor (GR) in health and disease. The text in black indicates the role of the GCs and the GR signalling pathway in the major organ systems within the body. The green text indicates the diseases and procedures within the clinic that routinely use GCs as a treatment. Whereas the blue text indicates the adverse outcomes in patients within the clinic who are exposed to high levels of GCs (Kadmiel and Cidlowski, 2013).

1.2.4 Glucocorticoid Receptor Signalling Pathway

In humans, the primary endogenous GC is cortisol, which is able to actively bind GR as a ligand (Marques et al., 2009). However, other non-glucocorticoid ligands are able to bind GR, including progesterone and progesterone synthetic derivatives, but with a relatively low affinity for GR compared to cortisol (Attardi et al., 2007).

In the absence of an active ligand, GR is predominantly present in the cytoplasm where it is bound to chaperone proteins in an inactive multi-protein complex (Kadmiel and Cidlowski, 2013; Newton, 2000). Upon ligand binding, GR undergoes a conformational change which allows it to dissociate from the multi-protein complex and translocate to the nucleus, where it can act as a transcription factor (TF) and regulate the transcription of specific target genes. Once in the nucleus, GR homodimers or two monomers bind specific DNA sequences known as GC response elements (GREs) and negative GREs (nGREs) respectively (Oakley and Cidlowski, 2013), to modulate transcription of the steroid responsive genes, in processes known as trans-activation or trans-repression depending on the outcome of transcription (figure 1.7) (Newton, 2000; Schlossmacher et al., 2011). GRα and GRβ can also form heterodimers, but these complexes lack transcriptional activity within cells and are thought to result in the inhibition of GR signalling (Koga et al, 2005). Additionally, it has been suggested that GRβ may compete directly with GRα, and act as an antagonist of GR activity by either competing for GREs within target genes, or by forming heterodimers with GRα as previously mentioned or even competing with TFs which are interacting with GRα itself, which leads to interference and inhibition of GRα signalling (He et al, 2015; Tissing et al, 2006). GREs are characterised by a 15 base pair (bp) consensus sequence: 5' – AGAACAnnnTGTTCT – 3' (Turner et al, 2010; Revollo and Cidlowski, 2009). Whereas the consensus nGRE sequence is: 5' – CTCC$(n)_{0\text{-}2}$-GGAGA – 3', which is a palindromic sequence but differs from the previously mentioned GRE consensus sequence. This is due to the presence of a variable spacer which ranges from zero to two nucleotides and also due to it being occupied by two GR monomers (Oakley and Cidlowski, 2013). Binding of GR to nGREs allows GR to repress the expression of certain transcripts, however despite the fact that it seems that the majority of the genes which are regulated by GCs and the GR signalling pathway are being repressed, very few nGREs have been identified in these target genes (Dostert and Heinzel, 2004; Surjit et al, 2011).

Alternatively, instead of this direct interaction with DNA, GR can regulate gene expression through non-genomic protein-protein interactions with other TFs, such as NF-κB, AP-1 and STAT (Marques et al., 2009).

GCs are also known to bind the mineralocorticoid receptor (MR) with a higher affinity than GR; therefore MR is occupied under basal conditions in the body, whereas the GR is only occupied by GCs during stress responses and in the presence of active ligand (Armario, 2006).

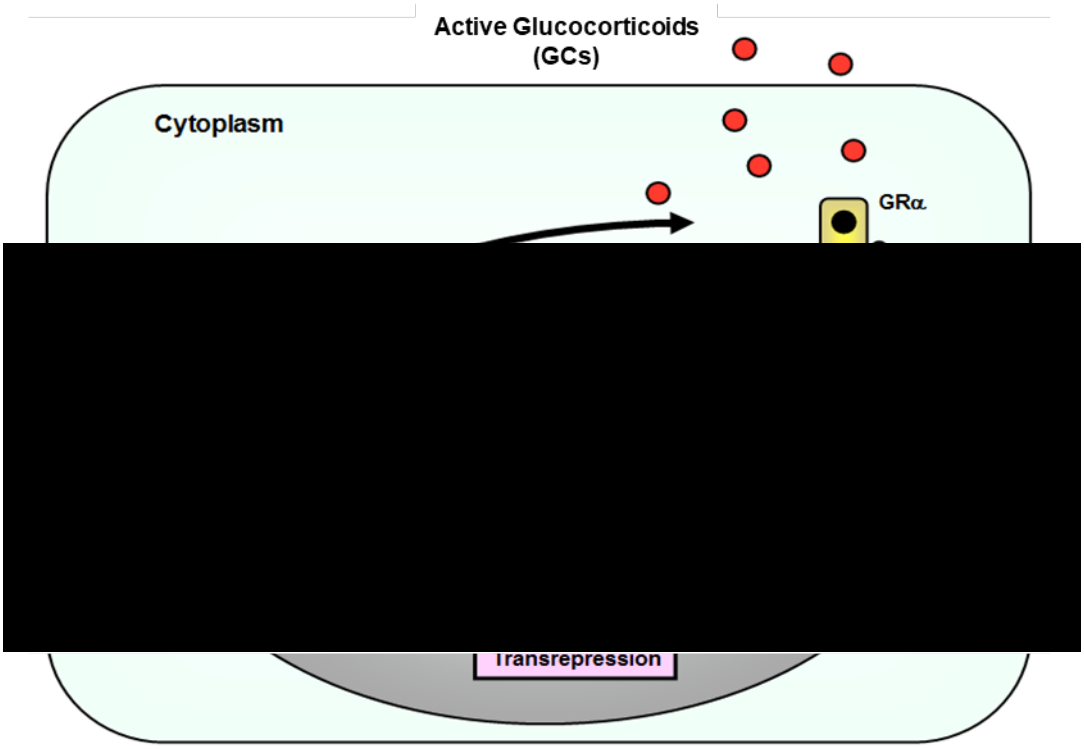


Figure 1.7. Diagram showing glucocorticoid receptor (GR) signalling. Upon binding to the active ligand, the glucocorticoid receptor (GR) is able to dissociate from its multi-protein complex within the cytoplasm and translocate to the nucleus. In the nucleus, GR can form homodimers and bind to glucocorticoid response elements (GREs) directly located in the promoter region of target genes. GR can also interact with other transcription factors (TFs) via protein-protein interactions, to either activate or repress the transcription of GC responsive genes known. This is known as trans-activation or trans-repression (Brain Immune, 2010).

11β-HSD2 which catalyses the conversion of cortisol to cortisone is mainly expressed in tissues which depend on MR activation by mineralocorticoids, such as the kidney. And as aldosterone and cortisol are both potent MR agonists, 11β-HSD2 activity is required at these sites to deactivate cortisol and prevent it from occupying MR (Hillier, 2007).

1.2.5 Co-factors of the Glucocorticoid Receptor

Effective GR-mediated stress signalling relies on the coordinated activity of numerous co-factors and chaperones, which fold GR into a mature state, regulate its affinity for cortisol, facilitate its translocation into the nucleus, and modulate GR-mediated transcriptional activation or repression of target genes (Sinclair et al, 2013). Many GR co-factors have so far either been identified to remodel chromatin or recruit the basal transcriptional machinery to the GRE sites within the promoters of GR target genes (Schlossmacher et al, 2011). The major corepressors identified for GR are NCoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoid and thyroid hormone receptor), amongst many others. These corepressors bind the receptor in the absence of active ligand and repress GR-dependent transcription by recruiting histone deacetylases to the transcriptional complex which results in the chromatin adopting a 'closed state', which does not favour transcription (Davies et al, 2011; Schlossmacher et al, 2011). Conversely, the coactivators interact with GR either via the AF-1 or AF-2 domains. Binding to the AF-2 domain is hormone dependent, and coactivators such as members of the p160 family, p300/cAMP response element-binding protein (CBP), Brahma-related gene 1 (BRG1), p300 CBP associated factor (PCAF), and vitamin D receptor interacting protein 205 (DRIP205) / thyroid receptor associated protein 220 (TRAP220), interact with this domain (Davies et al, 2011). AF-1 is a hormone-independent activation function located in the N-terminus of the receptor, but it is not conserved in sequence or structure among the NR family. The coactivators' motif necessary for binding to AF-1 has not yet been characterized. However, proteins such as members of the p160 family which include SRC-1 and PGC1, DRIP150, and the tumour susceptibility gene 101 (TSG101) have been shown to interact with this domain (Davies et al, 2011; Schlossmacher et al, 2011).

As previously mentioned, in the absence of hormone, GR resides predominantly in the cytoplasm of cells as part of a large multi-protein complex that includes chaperone proteins and immunophilins of the FK506 family (Oakley and Cidlowski, 2013).

An essential co-chaperone protein of GR is the FK506 binding protein 5 (FKBP5) (Bali et al, 2016). It is known that FKBP5 is up-regulated by several hormones, which include progesterone, androgens and potentially GCs in several different tissues and cell lines (Patel et al, 2016; Spijker et al, 2012). Additionally, along with FKBP5 being a co-factor of GR, it may also be targeted by the GR signalling pathway due to the presence of GREs within its promoter region. It is thought that genetic variations in the FKBP5 gene may result in an increased expression of FKBP5 within cells, leading to alterations in GR function and its signalling pathway (Spijker et al, 2012).

Healthy patients who do have these genetic variations of the FKBP5 gene exhibit GR resistance and a reduced negative feedback of the HPA axis (Spijker et al, 2012).

Another co-chaperone protein of GR is FKBP4, which is known to have specific expression patterns during the menstrual cycle (Yang et al, 2012). Knock down studies of FKBP4 in HESCs have led to a reduction of decidualization of HESCs *in vitro*, which demonstrated the important role of this protein within the endometrium (Yang et al, 2012). Other important co-factors and chaperones of GR include p23, p59, calreticulin, BCL2 Associated Athanogene 1 (Bag1), Hsp70, Hsp90 and Hsp27 amongst others (Sinclair et al, 2013; Newton, 2000).

1.2.6 Imbalance of Glucocorticoids and Disease

Imbalances in the levels of GCs due to dysregulation of the HPA axis or endogenous GC excess can lead to numerous pathological conditions.

A condition which results in elevated levels of GCs in the body is anorexia nervosa, which is a psychiatric illness characterised by severe self-imposed malnutrition. One of the manifestations of this condition is amenorrhea, which is thought to be a protective adaptation to prevent pregnancy taking place in times of inadequate nutrition. It is known that patients suffering from anorexia nervosa have over-stimulation of the HPA axis and a chronic stress response due to the presence of starvation and high levels of circulating GCs (Usdan et al, 2008). The acute infertility caused by this condition can lead to long-term infertility even after treatment takes place and patients resume a normal weight (Loucks, 2007). Additionally, it is also known that plasma cortisol levels are increased in patients with type 2 diabetes (Chiodini et al, 2007; Reynolds et al, 2010).

The primary diseases associated with dysregulated cortisol levels have very complex inter-related symptoms and etiology. Cushing's syndrome and Addison's disease are the most studied manifestations of adrenal gland dysfunction that result in altered cortisol levels.

1.2.6.1 Cushing's Syndrome and Addison's Disease

An elevation of GC levels within the body can lead to Cushing's syndrome, whereas a deficiency of GCs can lead to Addison's disease (Kadmiel and Cidlowski, 2013; Marques et al., 2009).

The symptoms of Cushing's syndrome are similar to those seen in the Metabolic Syndrome (MetS), which describes an assortment of closely related metabolic disorders including obesity, hypertension, insulin resistance, hyperglycaemia and hyperlipidaemia (Rose and Herzig, 2013). Addison's disease results in many non-specific clinical symptoms including fatigue, weight loss and hypotension (Kadmiel and Cidlowski, 2013).

Reproductive disturbances also occur in patients suffering from Cushing's syndrome, where males suffering from this disorder exhibit low plasma testosterone levels, low testosterone production rates and endogenous hypercortisolism (Smals et al, 1977; Vierhapper et al, 2000). However, no differences are seen in either testosterone production or levels in women suffering from Cushing's syndrome compared to control patients, which suggest that it is gonadal testosterone production which is suppressed by excess GC levels (Vierhapper et al, 2000). Furthermore, those suffering from this syndrome also have an increased risk of developing major depressive disorder, which is thought to be related to the high levels of cortisol levels present in circulation (Sonino and Fava, 2001; Sonino et al, 1998).

1.2.7 Altered Cortisol Metabolism

It has been found that impairments in cortisol metabolism may result in androgen excess within the body, which is observed in women suffering from Polycystic Ovary Syndrome (PCOS) compared to fertile women. The metabolism of cortisol mostly takes place within the liver and kidney, and occurs via several enzymes, such as 5α-reductase, 5β-reductase or through the 11β-HSD enzymes (Tsilchorozidou et al, 2003).

It was demonstrated by Tsilchorozidou and colleagues using 24h urine collections that an increase in 5β-reductase activity and a reduction in the 11βHSD-1 activity seemed to be responsible for the increase in cortisol metabolism seen in lean PCOS women. This resulted in a decreased negative feedback suppression of ACTH production due to a decreased conversion of the inactive cortisone to the active ligand cortisol. This ultimately leads to the over-stimulation of the HPA axis in order to compensate for the low levels of circulating cortisol at the expense of androgen levels (Tsilchorozidou et al, 2003).

Therefore this research demonstrates that more studies need to be conducted to investigate the effect of high levels of circulating cortisol in PCOS patients and whether altered cortisol metabolism may have a role in the clinical manifestations of the syndrome, such as infertility and menstrual disturbances.

1.2.7.1 Chronic Stress and the Effects on the Body

Chronic stress is known to be involved in the development and maintenance or exacerbation of numerous mental and physical health conditions. These include RA, asthma, cardiovascular disease (CVD), anxiety, depression, chronic pain, stroke and particular types of cancer (Slavich, 2016).

There has been considerable evidence that stress and hence GCs play a significant role in women's health, particularly within cancer development and progression. There has been both epidemiological and molecular evidence for an association between stress and the development of breast cancer in particular (Vilasco et al, 2011; Antonova, Aronson and Mueller, 2011). Along with progesterone and estrogen, GCs have also been found to regulate the physiology and pathology of breast tissue and furthermore GR is expressed within cells of the breast (Vilasco et al, 2011). It has been described that GR can exert anti-apoptotic effects and directly affect the cellular proliferation of MCF-7 breast cancer cells (Vilasco et al, 2011). However, results have been varied and it is thought that the type of stress, whether it be acute or chronic also influences the risk of developing breast cancer (Vilasco et al, 2011; Volden and Conzen, 2013).

1.2.8 Glucocorticoids and the Reproductive System

It is becoming more apparent that GCs play a significant role within reproduction in both the male and female reproductive systems. It is known that GC levels within the human body can lead to both repressive and enhancing effects on reproduction which is summarised in figure 1.8 (Whirledge and Cidlowski, 2013).

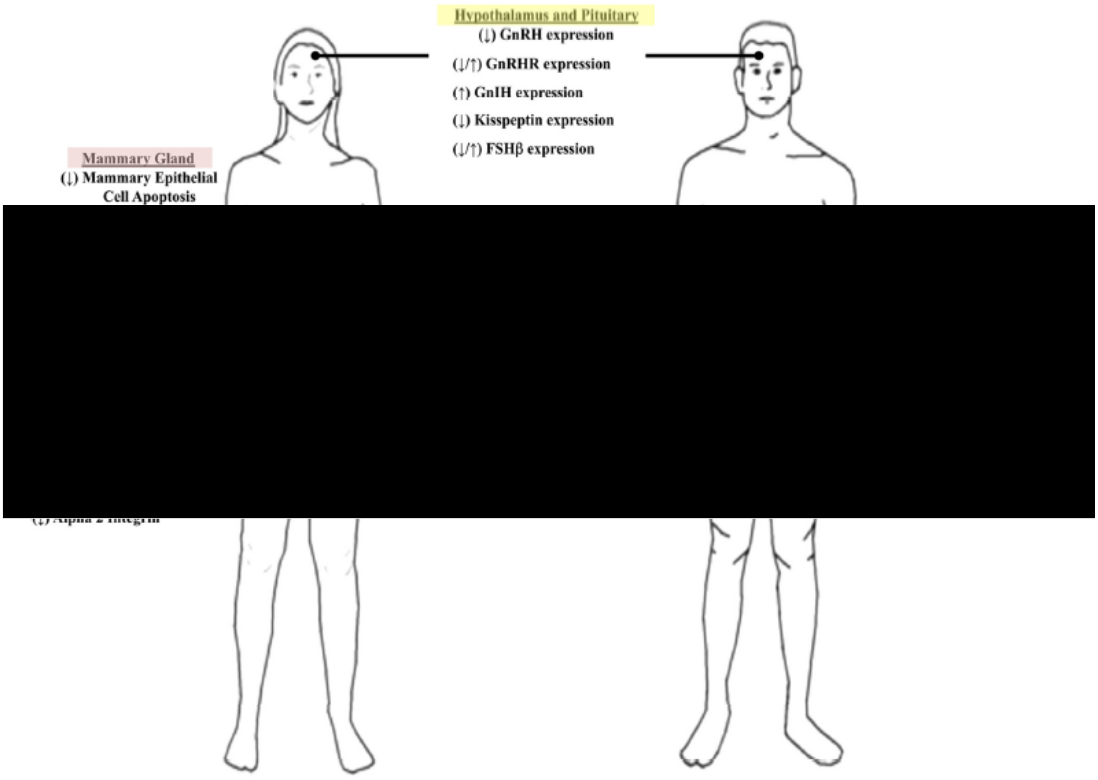


Figure 1.8. Diagram showing a summary of the role of glucocorticoids (GCs) in tissues of the reproductive tract and within the hypothalamus. GCs induce both repressive and enhancing effects in both males and females which have a significant effect on reproduction. Arrows pointing up indicate enhancing effects and arrows pointing down indicate repressive effects (Whirledge and Cidlowski, 2013).

In pregnancy, it is known that the activation of the maternal HPA axis and the consequent production of GCs are involved in the maintenance of homeostasis of many physiological processes– including the inflammatory and immune responses (S. D. Whirledge et al., 2015). The HPA axis is dramatically affected, with the axis becoming increasingly more active as pregnancy progresses, which results in a gradual rise in cortisol levels which reach a peak in the third trimester (Jung et al., 2011; Michael et al., 2013).

As it is has been suggested that GCs exert their effects within the reproductive organs and tissues, many studies have been conducted to elucidate a relationship between stress exposure and GC production with fertility in both males and females (Campagne, 2006).

Previous studies have focused on the effect of GCs and the HPA axis signalling pathway on female reproduction in particular, where it is known that chronic stress exposure can inhibit the synthesis and release of the gonadotropins. The luteinising hormone (LH) and follicle stimulating hormone (FSH) stimulate the gonads to produce the sex steroid hormones (S. D.

Whirledge et al., 2015). However, recent research suggests that GCs may also be having a direct effect on the human endometrium and ovaries (Whirledge, Senbanjo, and Cidlowski, 2015). A relationship has been found between fertility and the activity of the ovarian and endometrial 11β-HSD enzymes (Campagne, 2006). Smith and colleagues demonstrated that 11β-HSD2 was expressed within the human endometrium and myometrium, with a higher activity of 11β-HSD2 throughout the menstrual cycle compared to 11β-HSD1. 11β-HSD2 activity was also significantly higher during the secretory phase of the menstrual cycle compared to other phases. Furthermore, patients who were taking combined oral contraceptives (OCPs) had a significant reduction in the activities of both 11β-HSD isoforms (Smith et al, 1997). Granulosa cells isolated from patients undergoing IVF and embryo transfer (IVF-ET) have been cultured *in* vitro, and expression analysis revealed that ovarian 11β-HSD expression may be an important determinant and predictor of IVF success rates (Michael et al, 1993, 1995).

Nevertheless, due to the complex nature of the relationship between stress and fertility and the molecular mechanisms involved, the results obtained to date have remained inconclusive.

1.3 The Female Human Reproductive System

Human reproduction is a complex series of processes, where defects occurring at any step can lead to unsuccessful pregnancy. Some of the crucial steps which play a pivotal role in determine if a pregnancy is going to be successful are uterine receptivity, implantation of the embryo and endometrial remodelling(S. D. Whirledge et al., 2015). Numerous hormones and signalling pathways control these processes, which include the well characterised ovarian steroid hormones; estrogen and progesterone. Recent research suggests that GCs may also play a significant role throughout reproduction and pregnancy (Jung et al., 2011; Michael et al., 2013; S. D. Whirledge et al., 2015).

1.3.1 Physiology of the Reproductive System

The female reproductive organs consist of the ovaries, fallopian tubes and the uterus (figure 1.9). The fallopian tubes are located adjacent to the ovaries and connect directly to the uterus. The fallopian tubes are lined with ciliated epithelial cells and have an average length of 11cm (Briceag et al, 2015). At the ovarian end of the fallopian tube, there are projections present known as fimbriae, which are used to capture the ovum once ovulation has occurred, ensuring that it enters the fallopian tubes (Shaw, Luesley and Monga, 2011). The presence of the ciliated epithelial cells within the tubes also aids in the transport of the ovum to the uterus and the activity of these ciliated cells are essential within the secretory phase of the menstrual cycle, where the dysfunction of these cells can lead to infertility (Briceag et al, 2015). The uterus is composed of three major layers: the perimetrium, which is the peritoneal covering, the myometrium, which is the muscle layer and is also the thickest layer and finally the endometrium, which is the mucous membrane lining the cavity of the uterus and is a very specialised layer of tissue playing a crucial role during reproduction (Wilborn et al, 1983; Padubidri and Daftary, 2015). Lastly, the ovaries are extremely dynamic organs, where structural and functional changes can lead to far-reaching consequences on the fertility status of the female (Sargis, 2015). The ovaries contain a follicular compartment where the follicles develop into the ovum after receiving hormonal signals via the HPA axis during the menstrual cycle. Once the ovum has matured within the ovary, it is released during the process of ovulation (Nelson, Telfer and Anderson, 2013; Sargis, 2015).

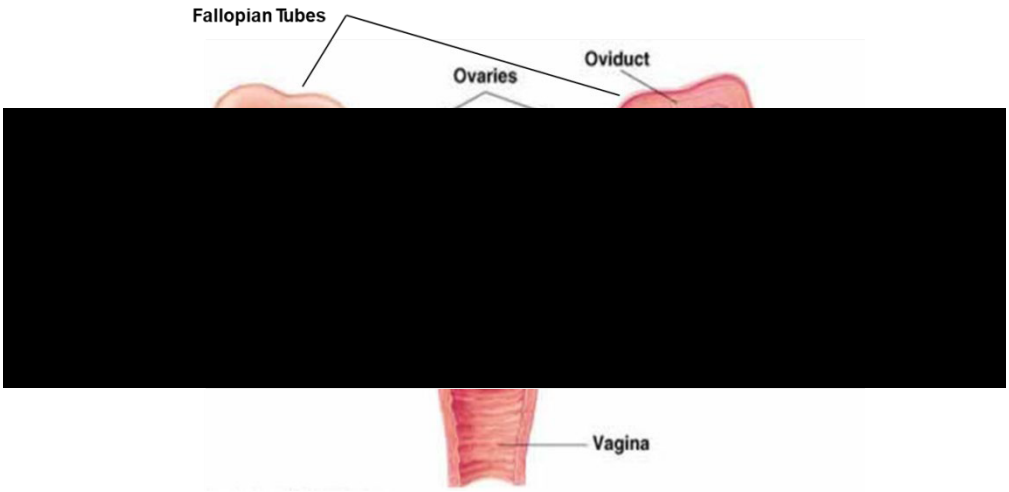


Figure 1.9. Diagram showing the structure of the female reproductive organs. (Adapted from Campbell's Biology, 5th edition, page 921 figure 49.9).

1.3.2 The Hypothalamic Pituitary Gonadal Axis

In addition to the previously mentioned HPA axis, there is an equally important axis within the body known as the hypothalamic-pituitary-gonadal (HPG) axis (figure 1.10). It is known that the HPG axis controls the production and release of several essential reproductive hormones; such as the luteinising hormone (LH) and the follicle stimulating hormone (FSH), which in turn control the production and release of the sex steroid hormones (Campagne, 2006; Whirledge and Cidlowski, 2010, 2013).

When the HPG axis is activated, the hypothalamus releases the gonadotropin-releasing hormone (GnRH), which activates a signalling cascade throughout the body (Whirledge and Cidlowski, 2010).

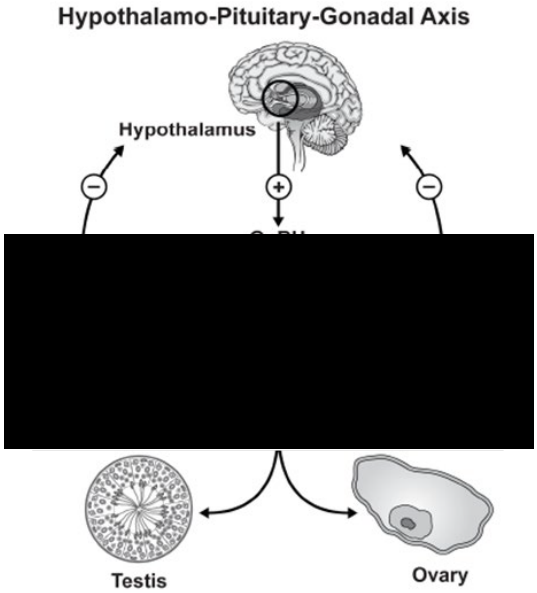


Figure 1.10. Diagram showing the Hypothalamic-Pituitary-Gonadal (HPG) Axis. The hypothalamus produces gonadotropin-releasing hormone (GnRH), which results in the anterior region of the pituitary gland producing luteinizing hormone (LH) and follicle stimulating hormone (FSH). In females, FSH and LH act primarily to activate the ovaries to produce estrogen and inhibin. The sex steroid hormone; estrogen, and FSH-stimulated inhibin feedback to inhibit the release of GnRH and pituitary secretion of LH and FSH (Whirledge and Cidlowski, 2010).

The release of GnRH stimulates the anterior pituitary gland to produce the gonadotropins; LH and FSH. This in turn stimulates the ovaries to produce estrogen in females (and the testes to produce testosterone in males) (Sargis, 2015). Additionally, the gonads also produce inhibin, which inhibits the production of the sex steroid hormones and therefore produces a negative feedback loop (Whirledge and Cidlowski, 2010, 2013).

The sex steroid hormones act as a negative feedback inhibitor of the production of GnRH by the hypothalamus (figure 1.10). The secretion and consequent synergistic actions of FSH and LH drives the maturation of the developing follicle within the ovary (Howles, 2000).

Studies have shown that the administration of CRH inhibits the secretion of GnRH and the consequent synthesis and release of LH (Young and Korszun, 1998; Viau, 2002). CRH neurones indirectly regulate the HPG axis via downstream GC production and directly regulate the axis via synapses located on GnRH neurones (Young and Korszun, 1998).

1.3.3 Reproductive Cycle

The reproductive cycle is controlled by specific levels of varying hormones, including the previously mentioned LH and the FSH released from the anterior pituitary gland which are ultimately under the control of GnRH (Howles, 2000).

Both LH and FSH are required for ovulation to take place, where FSH secretion controls the maturation process of the ovum and LH secretion controls the release of the mature ovum into the fallopian tubes (Shaw, Luesley and Monga, 2011). Estradiol is released at the beginning of every cycle and drives the proliferation of the endometrium and is therefore termed the proliferative phase (figure 1.11).This phase is required to prime the uterus for implantation by increasing the blood and nutrient supply (Mihm, Gangooly and Muttukrishna, 2011). Once there is a peak in LH concentration and ovulation has taken place at approximately day 14 of the menstrual cycle, the ovum is consequently released into the Fallopian tubes and the remaining follicle disintegrates into a 'corpus luteum', which becomes a source of the hormone; progesterone (Chabbert Buffet et al, 1998).

Ovarian hormones will then act on the endometrium, which is the tissue lining the cavity of the womb. The human endometrium is a highly specialised tissue, which undergoes cyclic changes each month termed the menstrual cycle. There are three phases of the menstrual cycle; proliferative, secretory and menses (or menstruation), which are characterised by varying levels of hormones (Figure 1.11) (Maybin and Critchley, 2012).

During this cycle, there are numerous changes of the endometrial morphology in response to varying levels of hormones. Firstly, the endometrium undergoes both proliferation and differentiation where it is primed for implantation. Implantation takes place in a crucial period termed the 'window of implantation'. Secondly, the endometrium undergoes degradation, and is finally regenerated approximately every 28 days (Chabbert Buffet et al, 1998).

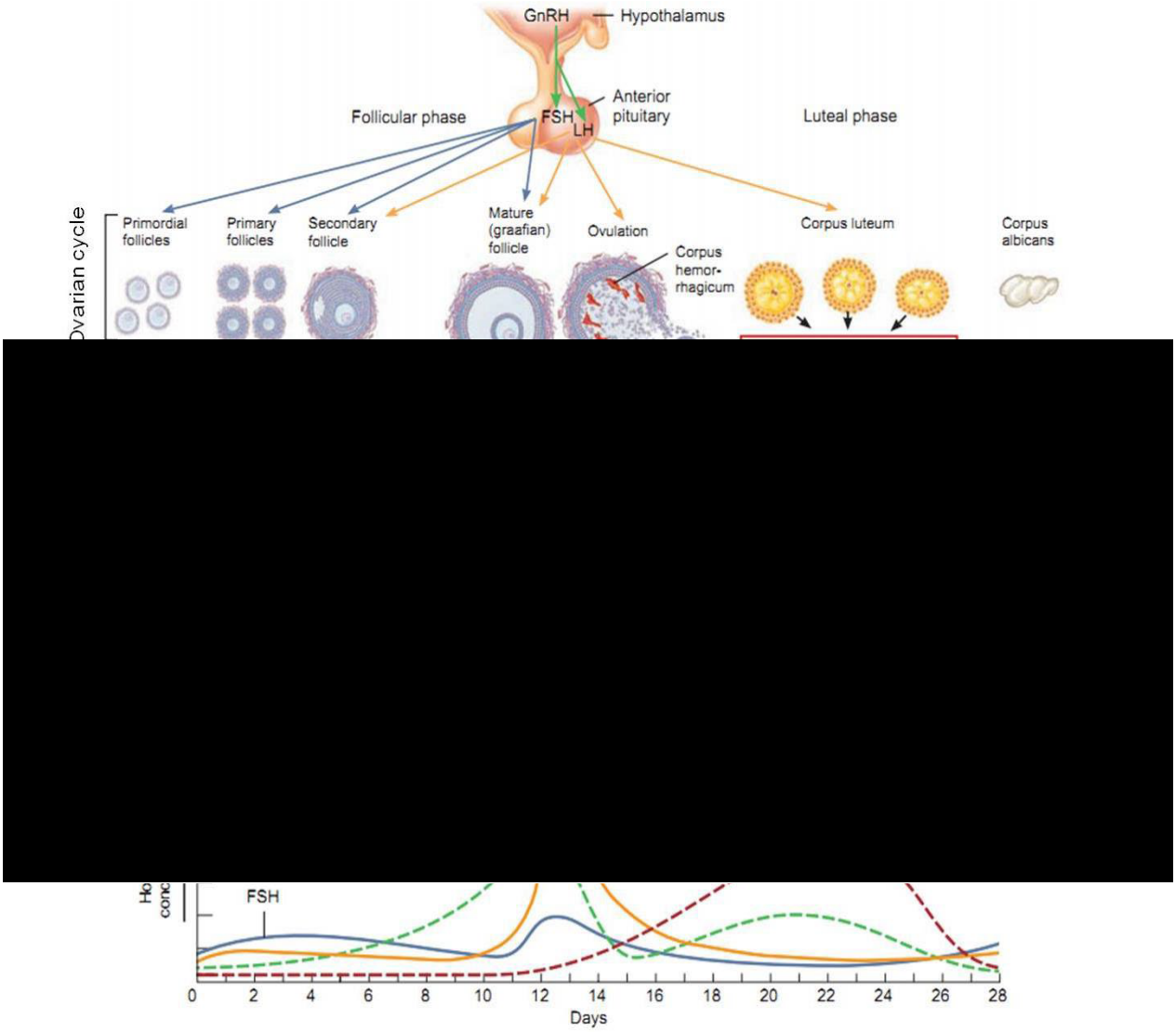


Figure 1.11. Diagram showing the reproductive cycle and changes in endometrial morphology in response to ovarian derived hormones and pituitary derived gonadotropins. The morphology of the endometrium is influenced by ovarian derived hormones; estrogen and progesterone and also pituitary derived hormones such as luteinising hormone (LH) and follicle-stimulating hormone (FSH). The proliferation of the endometrium tissue is driven by the presence of estrogen. Whereas the differentiation of the endometrium is driven by progesterone. FSH and LH signal to the ovary to release an egg and also provide a negative feedback mechanism for estrogen production (Adapted from De Mello, 2013).

Progesterone is responsible for the vascularisation and differentiation of the endometrium during the secretory phase until approximately day 23 of the menstrual cycle, where at this point the progesterone levels start to decline. This decline in progesterone leads to the constriction of the blood vessels and leads to menstruation. Days 20-24 of the cycle have been termed the 'window of implantation' as previously mentioned, which is where the endometrium is at its thickest and the epithelium is most receptive to the incoming blastocyst (Zhang et al, 2013).

Implantation involves the attachment, adhesion and the invasion of the blastocyst to the epithelial region of the endometrium. A process known as decidualization is crucial in order to support implantation, as this allows the blastocyst to embed in the decidualized stroma where it is then able to differentiate and grow. If implantation does not take place, the endometrium is broken down during the menstruation or menses phase which is characterised by a decrease in the levels of both LH and estradiol (Shaw, Luesley and Monga, 2011). Subsequently, the menstrual cycle is re-initiated and the above process is repeated.

1.3.4 Cellular Structure and Composition of the Human Endometrium

The endometrium contains a rich supply of blood vessels and is morphologically divided into two layers; the lower basal layer and upper luminal functional layer (figure 1.12) (Padubidri and Daftary, 2015). Both layers of the endometrium are multi-cellular with well-defined stromal and epithelial compartments (Shaw, Luesley and Monga, 2011). Adjacent to the myometrium, is the innermost layer of the endometrium, which is known as the basal layer, whereas the functional layer is the remaining top layer (Shaw, Luesley and Monga, 2011). The functional layer can be further divided into the stratum compactum and the stratum spongiosum, which contain the glands and stromal cells (Dallenbach-Hellweg, 1981). The functional layer is the layer which is re-built during every menstrual cycle after it is broken down and shed, whereas the basal layer differs as it remains during every menstrual cycle with minimal changes taking place. Crucially, the basal layer creates and supports the functional layer (Nair and Taylor, 2010; Aplin and Singh, 2008; Fabi and Asselin, 2014).

The major role of the endometrium is to establish and maintain pregnancy and makes up the inner glandular layer of the uterine wall, where it ultimately functions as the lining of the uterus (Shaw, Luesley and Monga, 2011). The endometrium is a highly dynamic tissue, responding to structural and biochemical changes which allow for endometrial receptivity (Aplin, 2007; Psychoyos et al, 1995). The endometrium responds to the HPG axis, reacts to cyclical changes of the levels of sex steroid hormones such as estrogen and progesterone, and also undergoes periodic proliferation, differentiation and breakdown (Shaw, Luesley and Monga, 2011; Jabbour et al, 2006; Salamonsen, 2003).

As mentioned previously, the vital role of the human endometrium is to coordinate crucial events which result in fertilisation, implantation and the maintenance of a successful pregnancy (Nikas et al, 1995; Gellersen et al, 2007). If implantation does not occur, the

dynamic remodelling of the endometrium takes place, which results in menses and the beginning of the next menstrual cycle as previously mentioned (Tabibzadeh et al, 1996).

In order for the endometrium to undergo recurrent structural changes which are crucial to the development and maintenance of pregnancy and ensuring optimal endometrial receptivity, it relies on cyclical ovarian function (Chabbert Buffet et al, 1998). Defects in endometrial receptivity can lead to impaired decidual responses and may even lead to sub or infertility.

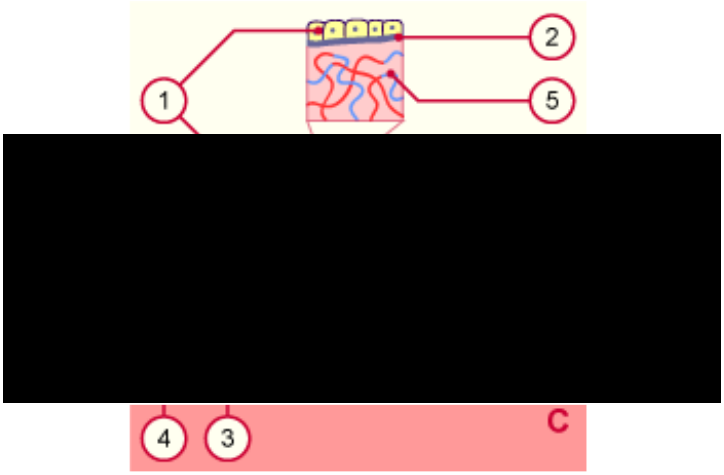


Figure 1.12. Diagram showing the structure of the endometrium. A = the functional layer of the endometrium, B = the basal layer of the endometrium, C = the myometrium lying beneath the endometrium, 1 = the single layered epithelium, 2 = the basement membrane and basal lamina of the single layered epithelium, 3 = the uterine glands, 4= the connective tissue and 5 = the blood vessels (Adapted from Human Embryology: Embryogenesis, 2017).

The endometrium consists of various distinct cell types, including the epithelial and stromal cells in addition to the glandular structures, which interact and communicate with one another throughout the menstrual cycle using the paracrine signalling pathway (Chabbert Buffet et al, 1998). The endometrial glands secrete proteins and cytokines, which play a pivotal role in signalling to the epithelium to prepare for blastocyst implantation (Zhang et al, 2013). The epithelial cells make up the surface of the endometrium and are the cells which make direct contact with the blastocyst during implantation (Shaw, Luesley and Monga, 2011). And finally, the stromal cells are located between the glandular and luminal epithelium (figure 1.13).

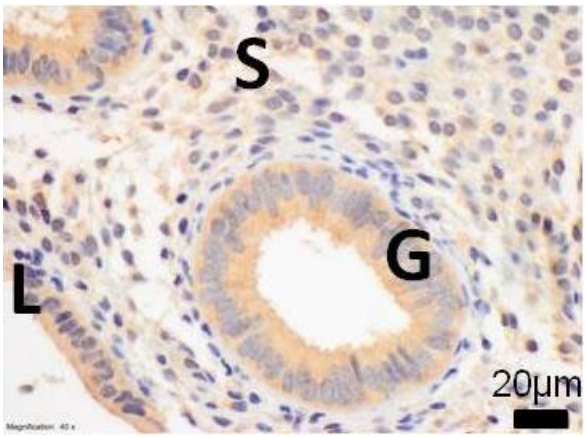


Figure 1.13. Immunohistochemistry (IHC) biopsy section showing the structural elements of the endometrium. The glandular region is indicated by (G), the stromal region by (S) and the lumen by an (L). Scale bar indicates 20 µm. Image was taken using a light microscope at x40 magnification.

1.3.4.1 Luminal Epithelium

The luminal epithelium is the primary site for the implanting blastocyst and contains both ciliated and non-ciliated cells, which differ in their number depending on the phase of the menstrual cycle and the cyclical changes of the sex hormones (Padubidri and Daftary, 2015). The morphology of these cells plays a crucial role in determining whether successful implantation will take place, due to alterations in cell adhesion (Shaw, Luesley and Monga, 2010).

1.3.4.2 Glandular Epithelium

The glandular epithelium is crucial during the menstrual cycle and undergoes changes throughout (Dallenbach-Hellweg, 1981). Post-ovulation, the endometrial glands alter their morphology and glycogen accumulates within the cells ready for the sustenance of the fertilised egg (Krantz and Phillips, 1962). This is under the control of the progesterone hormone, which is secreted and released from the corpus luteum within the ovary (Silberstein and Merriam, 2000). The corpus luteum remains after ovulation has taken place, where the granulosa cells within the wall of the follicle become luteinised and gland-like, consequently secreting both estrogen and progesterone (Maybin and Critchley, 2015; Shaw, Luesley and Monga, 2010).

During the proliferative phase of the menstrual cycle, where estrogen is the predominant hormone, the glands enlarge and become more complex with the cytoplasm and the mitochondrial components of the cells increasing, which allows the cells to undergo the crucial dynamic changes needed during the early secretory phase (Dockery et al, 1997). Conversely, during the secretory phase of the menstrual cycle, the predominant hormone is progesterone, which leads to increased glandular secretion (Chabbert Buffet et al, 1998). During this time, vacuoles and granules appear within the cytoplasm of the cells and substances such as glycogen and glycoproteins are secreted into the glandular lumen (Dockery et al, 1988).

1.3.4.3 Stroma

The stromal region contains many different cell types, which include fibroblasts, perivascular cells and leukocytes. The stromal cells have an important role within the endometrium in a process termed decidual transformation or decidualization. This process is where the stromal cells transform into decidual cells during the mid-secretory phase of the menstrual cycle (Mihm, Gangooly and Muttukrishna, 2011). This transformation involves the fibroblastic stromal cells undergoing a morphological change where they become much larger, rounded and globular (Ramathal et al, 2010). The decidualization process is essential for the successful implantation and consequent invasion of the embryo and therefore plays a vital role in the maintenance and development of pregnancy (Kliman et al, 2000). In the stromal region, there is also an accumulation of leukocytes during the receptive phase of the menstrual cycle, which are thought to play a role in controlling the invasion of the trophoblast within the endometrium and are also essential for immunosuppression (Pijnenborg, 2002, Pijnenborg et al, 1983).

1.3.4.4 Blood Vessels

The vasculature within the endometrium is very unique compared to other systems seen within the human body. This is due to the fact that the endometrial vasculature undergoes continuous phases of growth and reduction during the menstrual cycle (Rogers et al, 1996). The endometrium has a rich population of radial arteries, which emerge from the main arteries located within the myometrium. After the radial arteries cross the interface between the endometrium and the myometrium, they then branch into smaller basal and spiral arteries (Shaw, Luesley and Monga, 2011).

These smaller arteries supply different regions of the endometrium, with the basal arteries supplying the basal endometrium, whereas the spiral arteries supply the functional layer of the endometrium (Pijnenborg, Vercruysse and Hanssens, 2006). It is also known that the spiral arteries are affected by the cyclic hormonal changes which take place during the normal menstrual cycle, whereas the basal arteries remain un-affected (Ramsey et al, 1982).

1.3.4.5 Extracellular Matrix

The extracellular matrix (ECM) consists predominantly of collagen and proteoglycans which are produced by the previously mentioned fibroblastic stromal cells (Bilalis, Klentzeris and Fleming, 1996). The ECM has many functions, including acting as a structural frame for the tissue and also aiding in the interaction of various populations of cells present within the uterus, which takes place due to the expression of integrin's and various receptors (Aplin and Hey, 1995). Furthermore, the process of implantation allows the embryo to penetrate deeply into the maternal decidua and eventually to invade the endometrial spiral arteries. Implantation and formation of the placental villi are associated with remodelling of the ECM through tissue degradation by various proteinases, which include serine proteases, metalloproteinases and collagenases (Sillem et al, 1998).

1.3.5 Steroid Hormones and their Receptor Expression Levels in the Endometrium

The endometrium is targeted by the steroid hormones due to the presence and expression of their respective nuclear steroid hormone receptors (Maybin and Critchley, 2012). The presence of these steroid hormone receptors is fundamental for the action of the steroid hormones. The estrogen receptor (ER), progesterone receptor (PR) and the androgen receptor (AR) are expressed in the human endometrium. These receptors initiate signalling cascades which lead to altered expression levels of critical genes and proteins involved in reproduction. These receptors reside in the cytosol and are activated upon binding of their corresponding ligands, which results in receptor dimerization and translocation to the nucleus. Once the receptors are within the nucleus, they are able to bind regulatory sites within target gene promoters and either mediate or repress gene transcription (Critchley and Saunders, 2009).The presence of these receptors plays a crucial role in reproduction due to the fact that steroid hormones are known to be key regulators of normal uterine functions such as endometrial receptivity and implantation. In addition to ER, PR and AR, it is now known that the human endometrium also expresses GR and is therefore able to be targeted by the GCs (Bamberger, Milde-Langosch, Löning, and Bamberger, 2001). Therefore, the

key hormones known to be involved in reproduction include FSH, LH, estradiol, progesterone, androgens and more recently, GCs (Silberstein and Merriam, 2000)

FSH and LH play a primary role in ovulation, where they stimulate the ovaries to release estrogen which in turn has a crucial effect on the endometrium. These two hormones both have receptors, known as the follicle stimulating hormone receptor (FSHR) and the luteinising hormone receptor (LHR) which is a trans-membrane receptor found in the ovaries (Chabbert Buffet et al, 1998; Shemesh, 2001).

Progesterone (P_4) is a sex steroid hormone required for pregnancy and lactation and is produced almost entirely by the ovarian corpus luteum and the placenta (Stjernhom et al, 1996). Progesterone is synthesised from cholesterol and can be of ovarian, adrenal gland or adipose tissue origin. Progesterone is responsible for the differentiation of the endometrium during the secretory phase of the menstrual cycle. The concentration of progesterone is approximately 100 ng/mL at the beginning of the proliferative phase, where concentrations increase to their peak at approximately 350 ng/mL at day 21, before decreasing back to the starting concentration if pregnancy does not occur (Martin and Behbehani, 2006; Reed and Carr, 2018). Progesterone exerts its effects by binding to the PR, where there are at least two isoforms (PR-A and PR-B), which have distinct expression patterns (Gellersen et al, 2009). The expression of PR in the endometrium increases in the late proliferative phase of the menstrual cycle compared to the early proliferative phase and subsequently, its expression decreases during the mid-secretory phase and continues to decrease further in the late secretory phase (Ingamells et al. 1996).

The normal functioning of the endometrium requires the cyclic interaction of estrogen and progesterone and specific expression patterns of ER and PR. Estrogen mediates the growth of the endometrium and induces the expression of both ER and PR (Critchley and Saunders, 2009). After estrogen peaks during the proliferative phase of the cycle, progesterone and PR levels also start to increase. Conversely, progesterone counteracts the estrogen stimulation and down regulates both ER and PR (McCarty et al, 1979; Tseng et al, 1975). Progesterone also induces the expression of the 17β- hydroxysteroid dehydrogenase type 2 (17β-HSD2) enzyme within the endometrium, which converts the active ligand, estradiol to the inactive ligand estrone (Bulun et al, 2010). This results in a reduction in the local estradiol concentration, which is critical for optimal endometrial receptivity (Critchley and Saunders, 2009). The sequential supply of estrogen and progesterone and consequently the specific expression patterns of ER and PR are required for the successful development and maintenance of pregnancy.

Any disruption in this balance can lead to significant endometrial defects, such as implantation failure, which can result in various clinical pathologies (Bagot et al, 2000).

The endometrial epithelial and stromal cells both express ER and PR within their nuclei and the expression of both is at its highest during the mid to late proliferative phase (Lessey et al, 1988). The expression of ER significantly declines during the secretory phase of the menstrual cycle in both the epithelial and stromal cells. PR expression follows a similar pattern; however the stromal cells retain the expression of PR until the late secretory phase in the decidual cells only (Vienonen et al, 2004). This sustained expression of PR within the decidualized stromal cells allows for successful implantation (Critchley and Saunders, 2009).

Estrogen is also a major sex steroid hormone, where estradiol (E_2) is the most potent and pre-dominant estrogen present within the human body and is also the primary estrogen involved in controlling crucial processes within the endometrium (Bender, Buekers and Leslie, 2011). Estradiol is produced by the granulosa cells within the ovaries and is also produced by developing follicles and the corpus luteum. Estradiol is synthesised through the cholesterol pathway, where initially androstenedione is synthesised from cholesterol, which is consequently converted to estrone or testosterone. Testosterone, which is an androgen, can consequently be converted to estradiol via the aromatase enzyme (Young and McNeilly, 2010; Chabbert Buffet et al, 1998). It is known that estradiol drives the regeneration of the endometrium after menstruation during the proliferative phase of the menstrual cycle (Reed and Carr, 2018). Physiological levels of estrogen are approximately 150 ng/mL at the beginning of the proliferative phase of the menstrual cycle and this level gradually increases until day 13 of the cycle, where the concentration peaks at approximately 420 ng/mL. These levels consequently decrease until approximately day 17 of the cycle, before they increase again during the secretory phase, with levels peaking again at approximately 320 ng/mL during the 'window of implantation' (Martin and Behbehani, 2006).

The estrogen receptor (ER) has two major isoforms known as ERα and ERβ (Couse et al, 1997). Expression levels of both ER receptors increase from the proliferative phase to the early secretory phase of the menstrual cycle. However, during the mid-secretory phase the expression of ERα continues to increase whilst ERβ expression decreases. Conversely, during the late secretory phase, the expression of ERα starts to decrease whilst ERβ expression increases (Critchley and Saunders, 2009; Lecce et al, 2001). It is also known that ERα expression varies in response to progesterone and is down regulated during the 'window of implantation' (Lessey et al, 2002).

Abnormal expression of ER is known to be associated with various pathologies. For example, over-expression of ERα is seen in women with endometriosis and PCOS during the mid-secretory phase of the menstrual cycle, which leads to defects in endometrial receptivity (Lessey et al, 2002; Leon et al, 2008). The over-expression of ER suggests that these patients may have resistance to progesterone, which contributes to the clinical manifestations of these conditions, such as infertility. This effect is not seen in women suffering from unexplained infertility (Rey et al, 1998).

Another major group of sex steroid hormones, which play a crucial role within the human endometrium are androgens (Maybin and Critchley, 2012). Androgens are classically known as the male sex hormones, but it is now known that these hormones play a significant role in the maintenance of female fertility (Cloke et al, 2008). It has been reported that during a normal menstrual cycle, women have several androgens present in circulation, which include androstenedione, testosterone (T), dehyhdroepiandrosterone (DHEA) and dihydrotestosterone (DHT) (Maybin and Critchley, 2012). Even though DHT is only present at low levels in circulation, the cells of the endometrium contain the necessary enzymes which enable the conversion of the most abundant androgen; T to DHT, which is a more potent agonist of their corresponding receptor; AR (Maybin and Critchley, 2012; Andriole et al, 2004; Breiner et al, 1986). Androgens are produced and released by both the ovaries and the adrenal glands, but T and androstenedione are primarily produced by the ovaries (Maybin and Critchley, 2012). Androgens exert their effects by binding to AR, where the expression of AR has been described in the human endometrium (Mertens et al, 2001). AR is highly expressed in the stromal cells during the proliferative phase of the menstrual cycle in fertile women in comparison to the epithelial cells (Lu et al, 2006; Critchley and Saunders, 2009). Whereas, AR expression levels are very low during the mid-secretory phase of the menstrual cycle of normal cycling women in all endometrial cell types and is actually undetectable during the late secretory phase (Mertens et al, 2001). The exact molecular role of androgens and AR within the human endometrium remains elusive, but it has been suggested that AR may be required for the homeostasis of various crucial processes during reproduction, such as folliculogenesis (Kimura et al, 2007), and may play an important role in the differentiation of fibroblastic stromal cells into decidual cells through cytoskeletal organisation and cell cycle regulation (Cloke et al, 2008). The binding of androgens to AR, results in receptor translocation to the nucleus where it is able to bind to androgen response elements (AREs) located within the promoter regions of target genes, which allows AR to act as a TF and regulate their expression (Roy et al, 1999; Pandini et al, 2005).

There are several isoforms of AR, which include AR-A and AR-B which are the truncated and full length isoforms respectively (Gregory et al, 2001; Chang et al, 1988). The transcriptional activity of AR can also be aided by the recruitment of co-regulators (either co-activators or co-repressors) (Heemers et al, 2007). The importance of modulating androgen levels in females can be clearly seen in conditions involving abnormal androgen signalling, such as PCOS. Women suffering from PCOS have abnormally high circulating levels of androgens (hyperandrogenism) and high expression levels of AR during both the secretory phase of the menstrual cycle and the 'window of implantation' in particular, and therefore high levels of AR mediated gene expression (Maybin and Critchley, 2012; Norman et al, 2007; Apparao et al, 2002). The increased expression of AR seen in patients suffering from PCOS is thought to contribute to defects in endometrial receptivity, endometrial hyperplasia and high rate of miscarriages seen in this group of patients, in addition to the clinical manifestations of androgen excess such as hirsutism and obesity (Maybin and Critchley, 2012; Norman et al, 2007; Apparao et al, 2002).

Extensive crosstalk between the sex steroid hormone receptors is known to take place. Studies have shown that high doses of progesterone can not only result in the down-regulation of PR, but can also result in a decrease of AR and ER activity (Bardin et al, 1983; Raudrant et al, 2003). Additionally, it has been found that estrogen not only increases the expression of its cognate receptor, but also induces the expression of PR and AR (Fujimoto et al, 1994; Slayden et al, 2003).

Further research is needed to investigate and elucidate the complex role and potential crosstalk between the sex steroid hormone receptors and the glucocorticoid receptor within the human endometrium and the consequent effect on endometrial pathologies during the menstrual cycle. This will allow for an improved understanding of the role of the steroid hormones on fertility status and will allow for the development of novel clinical diagnostics and therapeutics.

1.3.6 Endometrial Receptivity

Endometrial receptivity is defined as the 'capacity for the uterine mucosa to facilitate successful implantation' (Cavagna, 2003). It is known that biochemical, molecular and structural events need to work in synchrony to ensure that the endometrium is at its most receptive.

The intricate crosstalk between different cell types within the endometrium allows optimal hormone and cytokine signalling to take place, and also allows for the specific expression of

adhesion proteins on the surface of the endometrium, which play an important role in implantation (Makker and Singh, 2006; Fatemi and Popovic-Todorovic, 2013). The expression of these adhesion proteins, peaks during the 'window of implantation' and therefore have been of great interest in fertility studies (Lessey, 2002; Goodison et al, 1999; Apparao et al, 2001).

Endometrial receptivity can be assessed using various imaging and visual platforms, such as Doppler ultrasonography and magnetic resonance imaging (MRI), which can evaluate endometrial thickness and texture and identify optimum conditions for receptivity in a clinical setting (Pierson et al, 2003).

Markers for endometrial receptivity include adhesion molecules, cytokines, growth factors and enzymes involved in the bioavailability of the steroid hormones among various other genes (Achache and Revel, 2006; Critchley and Saunders, 2009).

Despite this knowledge, more research is needed to shed light on the molecular mechanisms underlying endometrial receptivity and how these pathways can affect the critical process of implantation. This can be achieved using both primary cells isolated from human endometrial tissue and immortalised cell lines. However, gaining an in depth understanding of endometrial receptivity is particularly challenging due to the inability to use human blastocysts in experiments.

1.3.7 Implantation

The success of blastocyst implantation directly depends on endometrial receptivity, which has been previously described, the quality of the embryo and the communication between the embryo and maternal tissue (Simon et al, 2000).

The implantation of the embryo is known to be one of the most critical steps within reproduction and can be divided into distinct phases (figure 1.14). During the implantation process, firstly the blastocyst directly interacts and attaches to the receptive endometrium via numerous factors localised to the luminal epithelium, these stages are known as the apposition and attachment phases (Taylor and Gomel, 2008). There is a defined period within the menstrual cycle, where the endometrium is at its most receptive, known as the 'window of implantation', which lasts for approximately four days between day 20 and day 24, of the 28 day menstrual cycle (Psychoyos et al, 1995; Bergh and Navot, 1992). Secondly, the blastocyst adheres to the endometrial surface, which is characterised by cellular adhesion molecules such as integrin's, cadherin's and CD44 amongst other

molecules, and is termed the adhesion phase (Goodison and Tarin, 1999; Gonzales Amaro et al, 2005). The final phase of implantation is invasion, which is where the blastocyst passes through the epithelial basement membrane via apoptosis and the degradation of the ECM resulting in the invasion of the endometrial stromal region (Skrzypczak et al, 2007; Achache and Revel, 2006).

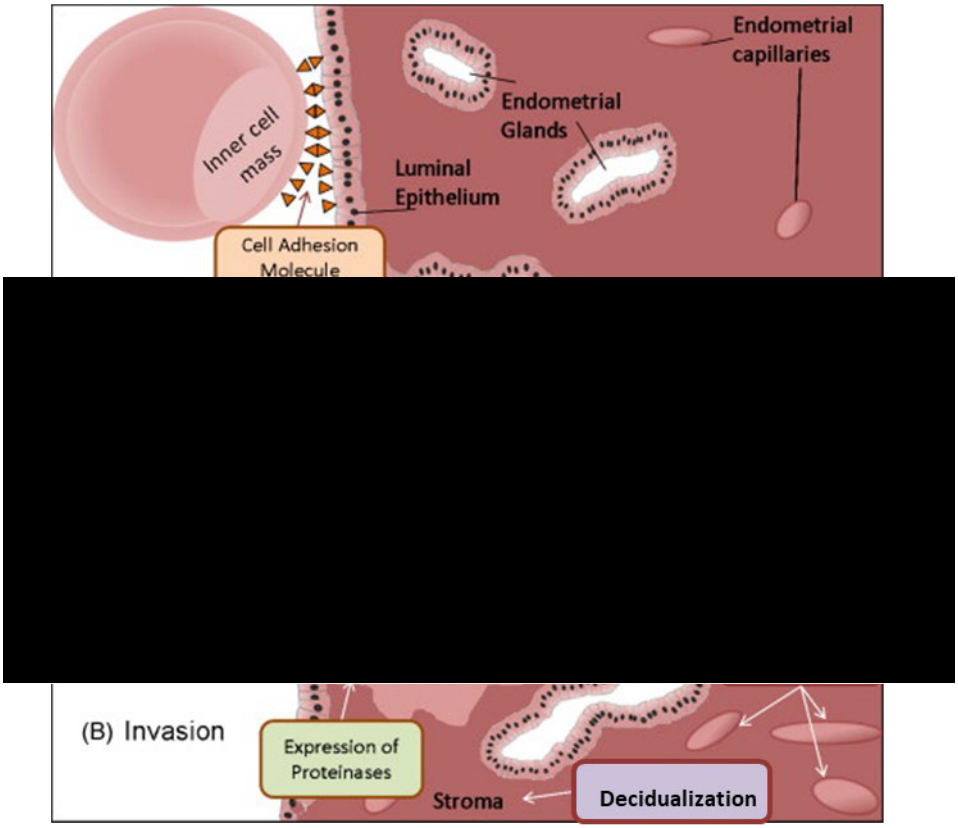


Figure.1.14. Diagram showing the sequential stages of implantation. (A) Adhesion/apposition—communication is established between the approaching blastocyst and the receptive endometrium, first via secreted molecules and then through the interaction of cell adhesion molecules displayed on both maternal and foetal cell surfaces. (B) Invasion—the blastocyst penetrates the endometrial epithelium, and invasive trophoblast cells migrate into the endometrial stroma region. (McEwan et al., 2009).

The stromal cells, which reside beneath the epithelium of the endometrium undergo a process termed decidualization in response to the invasion of the trophoblast. Decidua tissue supports the growth of the embryo, and initiates the production of the placenta, which becomes the interface between the foetus and maternal circulation (Denker et al, 1993; Aplin et al, 2000).

It is thought that delayed or an absent decidual response within the stromal cells may contribute to infertility, particularly within PCOS patients (Gregory et al, 2002) and to a

certain extent in endometriosis patients (Su et al, 2015). Moreover, defects in the expression of critical surface factors can lead to endometrial dysfunction, which is demonstrated in various pathologies including PCOS (Diamanti-Kandarakis et al, 2006).

Hence, implantation involves a specific interplay of both genetic and cellular signals which allow for the blastocyst to successfully interact and adhere to the endometrial epithelium to facilitate invasion and migration through the endometrial epithelium to the stromal region (Aplin et al, 2004; Lessey, 2002).

Further understanding of the molecular mechanisms involved in implantation would allow for the development of novel therapeutics for infertility and will also aid in the improvement of pregnancy success rates.

1.3.8 Decidualization of the Endometrium

The decidual transformation of the endometrium (also known as decidualization) is an essential process required for embryo implantation and the consequent establishment and maintenance of a successful pregnancy. Decidualization takes place immediately after the 'window of implantation' during the mid-secretory phase of the menstrual cycle, just after the post-ovulatory rise in progesterone levels and prepares the endometrium for the implantation of the blastocyst (Gonzalez et al., 2012; Jones, Salamonsen, and Findlay, 2002; Large and DeMayo, 2012).

Decidualization is mediated by the intracellular secondary messenger; cyclic adenosine monophosphate (cAMP) stimulating protein kinase A (PKA) and progesterone acting on an estrogen primed uterus, with low levels of androgens present (Dunn, Kelly, and Critchley, 2003; Gellersen and Brosens, 2003; Gellersen, Fernandes and Brosens, 2007; Gonzalez et al., 2012). Decidualization is characterised by the transformation, differentiation and proliferation of the endometrial stromal cells (ESCs) from their typical fibroblast-like mesenchymal phenotype into a much more rounded and globular epithelial-like phenotype, which is morphologically and biochemically distinct (figure 1.15) (Large and DeMayo, 2012; Tang et al, 1994; Salamonsen et al, 2003).

It has been demonstrated that impaired decidualization may have an impact on several reproductive disorders such as recurrent miscarriage, pre-eclampsia, PCOS and endometriosis (Ferrari et al, 1995; Zoumakis et al, 2000; Wang et al, 2004; Gonzalez et al, 2012; Quenby et al, 2002; Quinn et al, 2009).

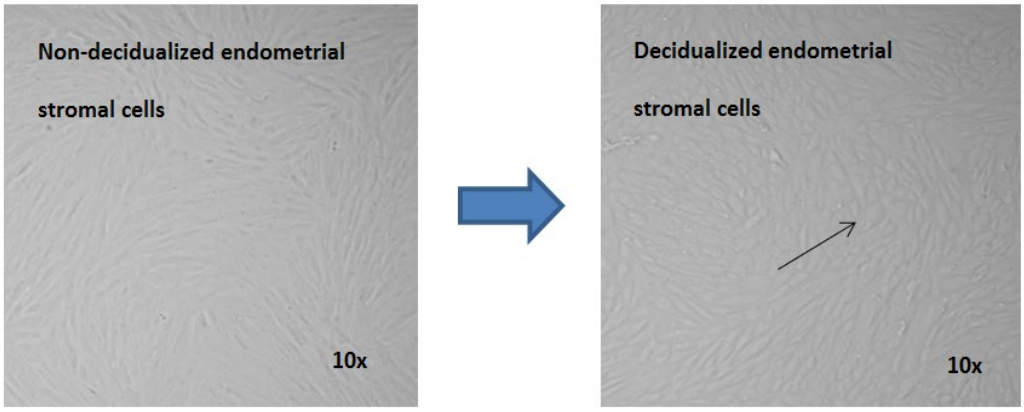


Figure 1.15. Figure showing example light microscopy images demonstrating the successful decidualization of endometrial stromal cells *in vitro*. Endometrial stromal cells (ESCs) isolated from fertile patients, successfully underwent *in vitro* decidualization with cAMP, to form a round phenotype i.e. epithelial-like phenotype (indicated by arrow), compared to the non-decidualized ESCs which are elongated i.e. fibroblast-like phenotype.

Decidualization is a complex morphological, biochemical and hormonal dependent process. Extensive research is needed to not only understand the molecular mechanisms underlying this crucial process, but also to identify how major signalling pathways within the endometrium may impact it.

1.3.8.1 Markers of Decidualization

The decidualization process is characterised by an increase in several key biological markers, which include decidual prolactin (dPRL) and insulin-like growth factor binding protein 1 (IGFBP-1) (Tamura et al, 2012; Telgmann and Gellersen, 1998). In addition to these highly investigated markers, decidualization can also be monitored using transforming growth factor β1 (TGFβ1) and E-cadherin (Telgmann and Gellersen, 1998).

Prolactin (PRL) is also known as the luteotropic hormone and is encoded by the human PRL gene located on chromosome 6 (Evans et al, 1989).

In addition to the decidua, prolactin is also produced by the pituitary, myometrium and breast (Gerlo et al, 2006). PRL has many roles in the human body, which include growth and development, endocrinology, metabolism, reproduction and immune regulation. The expression of PRL is under the control of the pituitary promoter and is the primary *in vitro* marker of decidualization, however decidual and pituitary derived PRL are identical in their structure (Telgmann and Gellersen, 1998).

IGFBP-1 is also known as placental protein 12, and is known to be one of the most abundantly expressed proteins in response to progesterone stimulation within the endometrium (Joshi et al, 1980; Tamura et al, 2012).

The expression of prolactin and IGFBP-1 at the gene and protein level in differentiating human ESCs depends upon elevated levels of cAMP and progesterone. There is strong evidence for the role of cAMP and its analogues in the decidualization of human ESCs *in vitro* (Dunn et al., 2003). *In vitro* experiments have shown that human ESCs in culture respond to cAMP more potently than progesterone to initialise decidualization (Brosens et al, 1999).

1.3.9 Infertility and its Causes

The National Institute of Clinical Excellence (NICE) defines infertility as 'the failure to conceive after regular unprotected intercourse for two years in the absence of known reproductive pathology' and one in seven couples worldwide, are known to be seeking help to achieve a successful pregnancy (NICE, 2012). Additionally, at least a quarter of couples are known to experience unexpected delays achieving a successful pregnancy, however, only half seek professional advice (Boivin et al, 2007; ESHRE Annual Report, 2011). Approximately one in five of these cases is known to be related to anovulatory disorders, and is therefore due to deficiencies in egg formation. Furthermore a proportion of the cases are due to ovulatory dysfunction, which means that successful ovulation is not occurring regularly. In addition to the recognised causes of infertility, there are other irregularities in the reproductive process which can also impair conception, such as the quality of gametes and the embryo which can lead to failure of fertilisation (Macer and Taylor, 2012). This demonstrates how processes both during and post-fertilisation can be affected by mechanisms which are largely unknown, ultimately leading to the failure of fertilisation.

It is known that *in vitro* fertilisation (IVF) is widely available for infertile couples within the UK, but only 25-30% of IVF treatments actually result in a live birth (Norwitz et al, 2001; Santos et al, 2003; ESHRE Annual Report, 2011). These failures during IVF are not due to failure of fertilisation, but conversely may be affected by other physiological mechanisms such as defects in implantation, endometrial receptivity and ovulatory disorders or may even be due to conditions such as endometriosis (Norwitz et al, 2001; Baart et al, 2007). Furthermore, there is growing evidence that these IVF failures may also be due to stress factors or the stress signalling pathway (Csemiczky, Landgren and Collins, 2000).

In addition to biological factors, there are also various environmental factors that can have an impact on fertility status. One environmental factor which has been extensively studied is smoking during early pregnancy (Soares et al, 2008). There is also growing clinical and epidemiological evidence that stress exposure may be playing a significant role on the fertility status of females (Akhter et al, 2016; Lynch et al, 2014). However, more research is essential to enable us to understand the underlying mechanisms involved in this process.

The ability of a female to establish a successful pregnancy is also known to decline with age (Balasch, 2010). Age is considered to not only be an important factor in the ability of women to spontaneously conceive, but is also known to play a significant role in the success rate of assisted reproductive technologies (ARTs), such as IVF (Wallace and Kelsey, 2010; Menken et al, 1986; Templeton et al, 1996). However, due to changes in lifestyle and culture, an increasing number of women are now choosing to conceive at an advanced maternal age (Crawford and Steiner, 2014). This may lead to a negative impact on IVF treatment and IVF success rates, as studies suggest that older women are significantly more likely to be diagnosed with unexplained infertility (UI) (Miller et al, 1999; Gleicher et al, 2006). There is currently no universally accepted definition for advanced reproductive age in females, but it has been suggested that women over the age of 35 years should be referred for further investigation after only 6 months of unprotected intercourse and the inability to conceive naturally, compared to the standard one year for women under the age of 35, where further investigations and treatment for infertility can take place at an earlier stage (NICE, 2012).

It is important for physicians to establish the cause of infertility and the inability to conceive, which can aid in the patient treatment plans, management and prognosis. Physicians look at the couple's history and a physical examination will take place to aid in the identification of factors which may be contributing to their infertility (Liu and Case, 2011; Crawford and Steiner, 2014; Dun and Nezhat, 2012).

In this context, it has been described that female factors, including ovulatory disorders, tubal factors, peritoneal factors, oocyte quality and uterine factors amongst others, account for approximately 40% of infertile cases presented to physicians (Balasch, 2010; Lindsay and Vitrikas, 2015; Török and Tamás, 2013).

1.3.9.1 Ovulatory Disorders

The menstrual cycle is defined as a cycle which lasts for 25-35 days, with ovulation occurring between day 10 and day 21 of the cycle (Shaw, Luesley and Monga, 2011). To test and assess ovulation and identify ovulatory disorders, both ultrasound and biochemical analysis can be used to track the follicle and measure crucial hormones and markers involved in ovulation respectively (Jose-Miller, Boyden and Frey, 2007). These include 'at home' urine luteinising hormone (LH) kits, serum LH measurement and mid-luteal serum progesterone levels to confirm ovulation has taken place (McLaren, 2012). WHO has categorised ovulatory disorders into three main groups, group 1 account for 10% of cases and is due to hypothalamic pituitary failure, group 2 account for 85% of cases and are due to the dysfunction of the hypothalamic pituitary ovarian axis and finally, group 3 account for 5% of cases and is caused by ovarian failure (Lindsay and Vitrikas, 2015). Women within group 1 normally present with amenorrhea, which is most commonly due to low body weight or excessive exercise, women in group 2 include those with PCOS and hyperprolactinemia, whereas women in group 3 can conceive only with oocyte donation and IVF (Lindsay and Vitrikas, 2015). Women with irregular menstrual cycles are considered to present a defect in ovulation, which can include anovulation. PCOS is the most common cause for anovulatory fertility, where approximately 90-95% of women attending fertility clinics who are suffering from anovulation suffer from PCOS (Sirmans and Pate, 2013).

Experiencing high stress levels on a daily basis, whether it is psychological or physical may also have an impact on ovulation. It has been found that those perceived to be experiencing high stress levels have lower mid-luteal progesterone levels and are more likely to suffer from anovulation (Schliep et al, 2015). Conversely, other research studies have suggested that women exposed to acute stressors may actually be induced to ovulate under optimal estradiol environments (Tarin, Hamatani and Cano, 2010). Further work needs to be carried out to identify the effect of both acute and chronic stress on ovulation and the menstrual cycle and its underlying molecular mechanism.

1.3.9.2 Tubal Factors

Once ovulation has taken place and an oocyte is released, it must be captured by the fallopian tube and transported to the uterus (McLaren, 2012). Any damage to the Fallopian tubes or tubal obstruction can result in tubal factor infertility. Additionally, post-surgical adhesive disease, a previous ectopic pregnancy or endometriosis can also result in tubal dysfunction and lead to infertility (Lindsay and Vitrikas, 2015). There has been an

increasing incidence of tubal factor infertility, mainly due to an increased number of sexually transmitted infections (STIs) being contracted, where chlamydia and gonorrhoea are the most common STIs to cause infections in the lower genital tract and lead to pelvic inflammatory disease (PID) (Dun and Nezhat, 2012). It is recognised that if there has been a previous diagnosis of PID, it may also be possible that tubal disease is present, as PID is known to be the major cause of tubal damage if diagnosed at a late stage or if it remains untreated (Haggerty et al, 2010). Therefore there is an increased risk of infertility in women diagnosed with PID which increases with both the number and the severity of the pelvic infections (Dun and Nezhat, 2012). Other causes of tubal factor infertility include STIs with no evidence of PID, use of intrauterine contraceptive devices, pelvic surgery and post-surgical adhesive disease, previous history of an ectopic pregnancy or a history of endometriosis (Lindsay and Vitrikas, 2015).

During an infertility assessment, tubal patency can be assessed via hysterosalpingography (HSG) which is offered to women with no clear risk of tubal obstruction or damage, sonohysterosalpingography (SHG), chlamydia serology tests (CAT) or a laparoscopic or hysteroscopic dye test which is offered to women with a risk factor for tubal obstruction or damage (Dun and Nezhat, 2012).

1.3.9.3 Uterine Factors

Uterine factors play a crucial role during conception, particularly during embryo implantation, but the prevalence of these factors amongst infertile females is relatively low compared to other factors affecting infertility. Uterine factors can impair implantation and therefore fertility due to distortion or deformity of the uterine cavity, either due to congenital uterine anomalies or due to the presence of adenomyosis, endometrial polyps, Asherman syndrome or adhesions and uterine fibroids (Hatasaka, 2011). These factors play a major role in infertility amongst females, especially in those who experience recurrent miscarriage, where 25% exhibit a minor uterine abnormality of some sort (Acien et al, 1993; Kupesic et al, 2001; Taylor and Gomel, 2008).

1.3.9.4 Failure of Fertilisation

Fertilisation is defined as the process resulting from the fusion of two parental gametes – the oocyte and the spermatozoon, which meet in the oviduct and initiate a cascade of events

which can lead to fertilisation and ultimately the development of pregnancy (Neri et al, 2014).

Due to tremendous improvements in ART, it is not uncommon for IVF to achieve success rates approaching 70% in some clinics (Kahyaoglu et al, 2014). However, successful fertilisation depends on many factors which include tubal function and the quality of the gametes (Swain and Pool, 2008; Mahutte et al, 2003). The tests to assess tubal patency and appearance (section 1.3.8.2) give limited information on tubal function, given that sometimes healthy tubes do not result in a successful pregnancy. Additionally, even though there are now widely used tests for sperm quality which include the hemizona assay or the competitive sperm binding assay (Lasiene et al, 2013), the quality of the oocyte which is largely responsible for regulating the majority of the molecular and cellular mechanisms required for fertilisation to take place, can be tested using a polar body biopsy (Swain and Pool, 2008; Montag et al, 2009). Furthermore, oocyte activation failure may be due to defects in a sperm-specific phospholipase C zeta, which is known to activate mammalian oocytes (Kashir et al, 2010). Taking all this into account, fertilisation failure is still occurring and only becomes apparent after ART, such as IVF and ICSI have been performed (Yanagida, 2004). Fertilisation failure can result in devastating emotional and financial consequences for the patients and also poses difficult challenges for the clinician (Kahyaoglu et al, 2014).

The gametes are extensively tested before ART cycles take place, in terms of both quality and potential, but this technique still renders poor outcomes with ICSI resulting in a fertilisation failure rate of 30% and a complete fertilisation failure occurring in approximately 3% of ICSI cases (Swain and Pool, 2008; Neri et al, 2014). Additionally, research suggests that fertilisation failure occurs in approximately 10% of IVF treatment cycles (Yanagida, 2004). Fertilisation failure observed in IVF is mainly due to sperm abnormalities, whereas in ICSI it is thought that oocyte activation defects are the most frequent cause of failure and interestingly, couples who unfortunately experience fertilisation failure have a 30% increased likelihood of recurrence in subsequent cycles (Kahyaoglu et al, 2014).

The treatments for UI are empiric and include expectant observation with timed intercourse and lifestyle changes. Treatments include clomiphene citrate, intrauterine insemination, and controlled ovarian hyper stimulation with intrauterine insemination, aromatase inhibitors, IVF and laparoscopy (Moradan and Ghorbani, 2009, Jose-Miller, Voyden and Frey, 2007).

It is thought that low expression of cell adhesion molecules in the endometrium of UI patients may result in infertility, such as integrin expression in the endometrial stroma,

which is known to differ between fertile and infertile patients (Moradan and Ghorbani, 2009). However, an improved understanding of the etiology of fertilisation failure is needed to assist in the counselling of patients and in the development and optimisation of fertilisation treatment.

1.3.9.5 Poor Embryo Quality

Studies have suggested that embryos which are involved in ART procedures may lead to an increased risk of pregnancy being affected by congenital anomalies compared to conceptions which have been spontaneous. It has been observed that pregnancies achieved after ART, have a slightly increased incidence of birth defects compared to natural spontaneous pregnancies (7% compared to 5%) (Hansen et al, 2005). This topic remains debateable due to the lack of a suitable control group and also due to low power of the studies (Bonduelle et al, 2002; Schieve et al, 2003).

There has also been work conducted to identify embryo chromosomal abnormalities in infertile couples and couples that have undergone IVF cycles with no success. Research suggests that infertile couples, especially those who have experienced failed IVF cycles have a higher rate of chromosomal abnormalities, which consequently affect embryo quality and the ability of a successful pregnancy taking place (Stern et al, 1999).

Embryo culturing techniques for IVF have greatly improved. Embryos are cultured to the blastocyst stage which allows more precise screening and selection of embryos of good quality (Shaw, Luesley and Monga, 2011). Even so, recent studies have shown that identifying genetically poor embryos which are therefore of a low quality, before IVF takes place, does not have any significant effect on IVF success rate (Blockeel et al, 2008).

Nevertheless, these results remain controversial and more research is needed to establish whether ART and embryo quality may be affecting pregnancy outcome.

1.3.9.6 Implantation Failure

Embryo implantation involves the interaction between the blastocyst and a receptive uterus which occurs in specific time period during the menstrual cycle known as the ‘window of implantation’ (Zhang et al, 2013). Successful implantation is essential to allow the establishment of pregnancy to take place. To date, the molecular mechanisms involved in implantation within the human endometrium are not fully understood. However, it is considered that the quality of the embryo and the reactivity of the uterus are among some of

the crucial factors which are significantly influencing the success of implantation (Zhang et al, 2013).

It is thought that implantation failure can either be referred to as cases who have no evidence at all of implantation and therefore no detectable HCG production or cases that have evidence of implantation and therefore detectable HCG production but it did not proceed beyond this stage (Timeva, Shterev and Kyurkchiev, 2014).

Implantation failure is known to be a major cause of female infertility during ART and also natural conceptions, where it accounts for 75% of pregnancy losses (Zhang et al, 2013). Even though, methods are now in place to identify good quality embryos for IVF cycles, unfortunately only 25% of embryos undergo successful implantation (Grochowski et al, 2001). Recurrent implantation failure during IVF is defined as 3 failed IVF cycles, or the transfer of ten or more good quality embryos with no successful outcome, which may be due to inadequate uterine receptivity or poor embryo quality (Simon et al, 2000).

Several non-invasive procedures have been used to assess uterine receptivity to determine if successful implantation is likely, including ultrasonography of the endometrium to evaluate not only the thickness of the endometrium but to also identify the optimum time and conditions for implantation to take place (Steer et al, 1995; Quenby et al, 2002).

Additionally, research suggests that late implantation can also result in early pregnancy loss and consequently affect fertility status (Wilcox et al, 1999).

Environmental, metabolic and genetic factors may be affecting successful implantation, where several genes and environmental exposures have been shown to be related to implantation and the uterine receptivity (Lessey, 2002; Kao et al, 2003).

1.3.10 Polycystic Ovary Syndrome

Polycystic ovary syndrome (PCOS) is the most common heterogeneous, heritable endocrine disorder which affects 6-10% of the female population of reproductive age (Barthelmess and Naz, 2014; Dennedy, Smith, O'Shea, and McKenna, 2010; Sirmans and Pate, 2013). The Rotterdam criteria is currently used to diagnose PCOS, which identifies the predominant phenotypic characteristics of the syndrome to be ovulatory dysfunction, whether it be infrequent or irregular ovulation or absence of ovulation (anovulation), clinical or biochemical indications of hyperandrogenaemia and finally polycystic ovaries as seen in figure 1.16, which can be observed using ultrasound investigation and are defined as 12 or

more follicles in each ovary measuring 2-9 mm in diameter and/or increased ovarian volume (>10 mL) (The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004; Barthelmess and Naz, 2014; Sirmans and Pate, 2013; Legro et al, 2013). The Rotterdam criteria states that two of these three phenotypes have to be present before diagnosis can be confirmed (Dennedy et al., 2010).

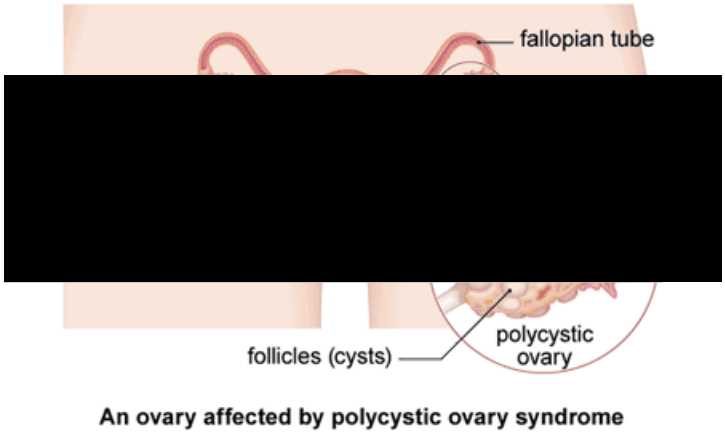


Figure 1.16. Figure showing the morphology of an ovary affected by Polycystic Ovary Syndrome (PCOS) (Bupa, 2016).

PCOS patients are also known to suffer from metabolic issues, which include obesity, hyperinsulinemia, dyslipidaemia, metabolic syndrome and type 2 diabetes (Tan et al, 2010; Ciampelli et al, 1999). This is in addition to the clinical manifestations of hyperandrogenaemia, which include hirsutism, acne and alopecia (Barthelmess and Naz, 2014). Menstrual disturbances such as oligo-menorrhea, amenorrhea and prolonged menstrual bleeding are often also observed in these patients (Sirmans and Pate, 2013).

It is important to note that there are several disorders which have similar clinical manifestations to PCOS, which include the previously mentioned Cushing's syndrome, hyperprolactinemia and congenital adrenal hyperplasia (Barthelmess and Naz, 2014).

Women, who are diagnosed with PCOS, can be sub-divided into either ovulatory or anovulatory PCOS patients (ovPCOS or anPCOS respectively). PCOS is known to be the primary cause of menstrual irregularity and anovulatory infertility amongst women (Lopes et al, 2011). Epidemiological data shows that approximately 90% of cases of anovulation are due to PCOS and 40% of all PCOS patients are thought to be affected by infertility (Barthelmess and Naz, 2014; Sirmans and Pate, 2013). Interestingly, it has been suggested that miscarriage rates particularly during the first trimester of pregnancy are significantly higher in PCOS patients compared to fertile patients (Legro et al, 2007).

Studies have shown that the endometrium of PCOS patients have developmental abnormalities when compared to the fertile endometrium. There is known to be altered expression levels of the steroid hormone receptors, such as ER and PR (Margarit et al, 2009; Maragarit et al, 2010; Gonzalez et al., 2012). Moreover, infertility associated with PCOS has been linked to the elevated levels of androgens present locally within the endometrium and in circulation, along with elevated expression levels of the androgen receptor (AR) (Gonzalez et al., 2012). Numerous studies have shown that it is critical that androgen levels remain low to allow successful decidualization to take place (Gonzalez et al., 2012; Maybin and Critchley, 2012).

In addition to the altered expression of the sex steroid hormone receptors within the endometrium of PCOS patients, there is also known to be altered expression of cell cycle regulators, apoptosis modulators and growth factors during decidualization (Gonzalez et al., 2012). Therefore it has been proposed that hyperandrogenaemia, which causes the primary symptoms of PCOS may be having a detrimental impact on uterine receptivity, decidual transformation and key regulatory genes and networks, which may contribute to the increased incidence of miscarriages and infertility seen within this group (Gonzalez et al., 2012; Maybin and Critchley, 2012). Recent research conducted by the Reproductive Biology and Gynaecological Oncology (RBGO) research group at Swansea University has identified altered expression of Wilms tumour suppressor (WT1) gene in PCOS patients, which is known to be expressed during the window of implantation and may have a role in the decidualization process (Gonzalez et al, 2012; Makrigiannakis et al, 2001). Gonzalez and colleagues demonstrated that WT1 expression within the stromal compartment of the endometrium of PCOS patients is significantly lower compared to fertile patients and furthermore, WT1 expression is regulated by the presence of androgens *in vitro*. This led to the hypothesis that elevated levels of androgens lead to a disruption of the regulatory balance between AR and WT1 within the endometrium of PCOS patients (Gonzalez et al, 2012). These studies suggest that a better understanding of the role of the hormone receptors and their corresponding ligands is needed to shed light on the mechanisms involved in this pathology and their effect on key regulatory genes within the endometrium, which may lead to the development of clinical interventions and novel therapeutics (Maybin and Critchley, 2012).

Despite the fact that PCOS is a highly prevalent condition within the Western World, with an increasing incidence, the etiology of the disorder remains unknown. Research suggests that it may be a familial genetic syndrome caused by a combination of both genetic and environmental factors (Barthelmess and Naz, 2014).

There is currently no cure for PCOS, but there are several lines of treatments for patients. The first line of treatment is the combined oral contraceptive pill (OCP), which is given to patients who do not wish to become pregnant (Macut et al, 2015). The OCPs are able to regulate the menstrual cycle and reduce the levels of androgens present within the body leading to a clinical improvement of hirsutism (Barthelmess and Naz, 2014). In addition to OCPs, diet alterations, weight loss and regular exercise are also recommended, as they can help reduce the chronic symptoms associated with this syndrome (Spritzer, 2014).

In addition to the previously mentioned treatments, clomiphene citrate is the primary choice of drug for ovulation induction in women suffering from PCOS. Clomiphene citrate is a partially selective ER modulator and results in an ovulation rate ranging from 70-85% per cycle (Sirmans and Pate, 2013). Additionally, treatment with metformin, which is an oral biguanide insulin-sensitizing agent, has been shown to be beneficial in reducing hyperandrogenaemia and hyperinsulinemia which are commonly seen in PCOS patients (Stadtmauer et al, 2002). Metformin also allows patients to undergo normal menses and be able to maintain pregnancy due to its role in regulating the menstrual cycle and ovulation (Jose-Miller, Boyden and Frey, 2007). Therefore the use of metformin in PCOS patients who are undergoing IVF provides great benefits (Stadmauer et al, 2002) Additionally, other interventions include the use of letrozole, which is used to stimulate ovulation in women suffering from PCOS (Hu et al, 2018) and also the administration of gonadotrophins and surgery with laparoscopic ovarian drilling to obtain normal ovulatory cycles and reduce the risk of ovarian hyper-stimulation and multiple pregnancies (Lebbi et al, 2015).

1.3.11 Endometriosis

Endometriosis is a debilitating chronic inflammatory disorder known to affect approximately 6-10% of women of reproductive age and 25-50 % of infertile women (Bulletti et al, 2010; Hickey et al, 2014; Monsivais et al, 2016). Endometriosis is an estrogen dependent disease which is characterised by functional abnormalities of the eutopic endometrium of women with endometriosis compared to normal endometrium (Liu and Lang, 2011) and the presence of ectopic abnormalities in the pelvic region which can be visualised using ultrasound/MRI investigation. These abnormalities include endometriomas due to endometrial glands and stromal cells being found outside of the uterine cavity (Hickey et al, 2014). These ectopic cells are primarily found in the pelvic region of these patients, but can also be found in the bowel and diaphragm amongst other areas (Hickey et al, 2014). These cells are still under the influence of the steroid hormones during the menstrual cycle and therefore respond similarly to the cells residing in the uterus. This leads to the primary symptoms of this

disorder, such as chronic pelvic pain, bladder and bowel pain, dysmenorrhoea, dyspareunia and sub-fertility (Kennedy et al, 2005; Farquhar et al, 2007). Endometriosis can therefore cause adverse effects on personal relationships due to an accumulation of these symptoms and can also affect a person's quality of life resulting in psychological distress which has a significant impact on a patient's quality of life (Hickey et al, 2014).

Similar to PCOS patients, primary endometrial stromal cells (ESCs) isolated from patients suffering from endometriosis also have a reduced capacity to undergo successful decidual transformation and secrete lower levels of the key decidual markers compared to fertile patients (Klemmt et al, 2006; Yang et al, 2012).

The current diagnostic method for endometriosis involves undergoing a laparoscopy and pathological examination of the lesions (Monsivais et al, 2016). A non-invasive diagnostic test for this disease would be very useful, however even though novel biomarkers have been proposed, such as CA-125, which can sometimes be elevated in women suffering from endometriosis, however none have yet been shown to be consistently clinically useful (Chen et al, 1998). Moreover, even though eutopic endometrium is different in women suffering from endometriosis compared to fertile women, there is still not yet a standardised endometrial biopsy test which diagnoses endometriosis clinically (Hickey et al, 2014).

The etiology of endometriosis remains unknown, however it is thought that both environmental and genetic factors may play a role. It is known that having a sister with endometriosis, increases the risk of developing the disease by five-fold (Monsivais et al, 2016). A known risk factor for which is associated with the development of endometriosis is progesterone resistance and it may also be associated to other conditions, such as chronic fatigue, autoimmune disease, asthma and ovarian cancer (Hickey et al, 2014; Yang et al, 2012).

Numerous studies have been conducted in the hope of developing a successful therapy for endometriosis, but options for clinicians are still limited (Klemmt et al, 2006). Current treatment methods for endometriosis include pain management, OCPs, gonadotropin-releasing hormone (GnRH) agonists and aromatase inhibitors (Torres-Reverón et al, 2018). These hormonal treatments result in the patient being infertile due to an inhibition of ovulation. Surgery can also be pursued, where laparoscopy can be used to confirm and identify the extent and severity of endometriosis (Hickey et al, 2014) and the removal of endometriomas can aid in pain reduction and the restoration of fertility status (Grzechocinska and Wielgos, 2012). However, as endometriosis is a chronic disease, prospective data has indicated that recurrence after surgery occurs in 10-50% of cases one year post-surgery, with this number increasing over time (Hickey et al, 2014).

There is a crucial need for research to aid in the development of novel and effective treatments and diagnostics for endometriosis, especially treatments which will aim to target altered molecular signalling pathways in these patients (Klemmt et al, 2006).

1.3.12 Unexplained Infertility

Unexplained infertility (UI) is diagnosed when all standard diagnostic tests used to identify infertility, such as ovulation assessment and semen quality assessment or investigative laparoscopy, show no reproductive pathology which may be causing the infertility in either partner (Lindsay and Vitrikas, 2015). It is thought that approximately 25% of infertile couples are suffering from UI with an etiology which may be multifactorial (Jose-Miller, Boyden and Frey, 2007).

As there is no apparent dysfunction identified using standard diagnostic tests, there is currently no standard treatment for UI. The clinical recommendation for women suffering from UI is that they benefit from intrauterine insemination or clomiphene citrate therapy (Jose-Miller, Boyden and Frey, 2007). Physicians also suggest that if couples are affected by UI, and have been trying to conceive naturally for a number of years then ART could be offered, however the benefit of IVF has not been proven in patients with UI (Lindsay and Vitrikas, 2015).

It has been suggested that UI may be due to endometrial factors such as defects in receptivity or implantation as the endometrium of women suffering with UI may have abnormalities in the distribution of certain ECM glycoproteins resulting in endometrial defects leading to implantation failure (Bilalis, Klentzeris and Fleming, 1996). However, no clinical biomarkers have currently been identified to aid in the diagnosis of UI.

1.3.13 Stress and Infertility

It has been well documented in both males and females that increased stress levels may lead to the inhibition of reproduction (Schliep et al, 2015; Akhter et al, 2016; Bhongade et al, 2015). It is known that a person's ability to adapt after acute stress exposure promotes subsequent reproductive success and any dysregulation of this adaptive response can result in maladaptive changes such as long-term infertility. However, the relationship between stress and infertility remains to be a controversial topic (Lynch et al, 2014).

It is known that the HPA axis and the consequent production and release of cortisol influence hormones which have a major role in reproduction, which include LH and FSH (Whirledge and Cidlowski, 2013; Whirledge and Cidlowski, 2010). This interaction may be due to similar neurotransmitters within the hypothalamus controlling both stress and reproduction (Campagne, 2006).

It has been found that those suffering from major depression, post-traumatic stress disorder (PTSD) and anorexia nervosa are characterised by increased production and secretion of cortisol into circulation, a dysregulated HPA axis and the suppression of fertility (Young and Korszun, 2002). One of the diagnostic criteria of anorexia nervosa is amenorrhea, which is thought to be a protective mechanism initiated by the stress response which prevents successful pregnancy taking place (Usdan, Khaodhiar and Apovian, 2008).

Additionally, recent studies have described that the majority of male soldiers suffering from PTSD reported erectile or ejaculatory dysfunction (Green, 2003; Hirsch, 2009). Additionally, soldiers that participate in stressful exercises or are suffering from PTSD have reduced levels of testosterone and military veterans have an increased risk of being infertile or take a significantly longer time to conceive (Kobeissi et al, 2008; Mulchahey et al, 2001).

Moreover, it has been found that infertile women have higher levels of circulating cortisol compared to fertile patients (Csemiczky, Landgren, and Collins, 2000). Delayed puberty has also been observed in elite athletes including runners and gymnasts, as well as in girls suffering with anorexia nervosa who all display elevated levels of serum cortisol (Luger et al, 1987; Weimann et al, 1999; Munoz and Argente, 2002). Additionally, in athletes, the levels of GCs present within circulation are negatively correlated with menstruation frequency (Usdan, Khaodhiar and Apovian, 2008). Nevertheless, even in healthy girls who were not athletes, there remains to be a strong correlation with high GC levels and delayed puberty (Shi et al, 2011).

The molecular mechanisms underlying how acute or chronic stress may be affecting fertility status by altering the production and release of the crucial sex hormones and the expression of their corresponding receptors remains unknown.

1.4 The Reproductive System and the Glucocorticoid Receptor

1.4.1 Expression of the Glucocorticoid Receptor in the Endometrium

It was demonstrated by Bamberger and colleagues using immunohistochemistry (IHC) that the glucocorticoid receptor (GR) is expressed within the normal cycling human endometrium, specifically within the stromal region and with higher expression levels being observed during the menstruation and proliferative phases of the menstrual cycle (Bamberger et al, 2001; Maybin and Critchley, 2012).

The expression pattern of GR within the endometrium is evidently distinct from the expression patterns of both ER and PR (Bamberger et al, 2001). As seen in figure 1.17, menstruation is characterised by an increase in the concentration of GCs, which suggests that they may play an important role in menstrual bleeding and endometrial repair (Maybin and Critchley, 2012).

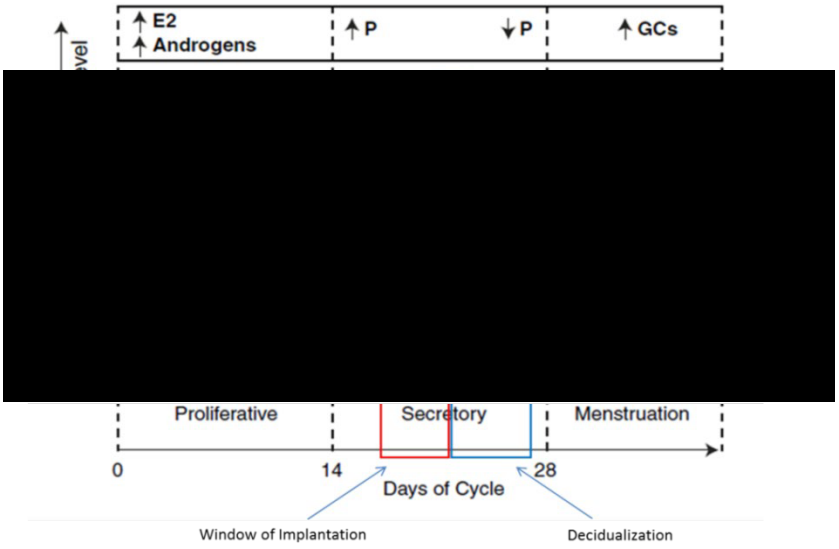


Figure 1.17. Diagram showing the phases of the menstrual cycle and the corresponding sex steroid hormone levels and their functional impact on endometrial tissue. The red box indicates the window of implantation (days 20-24), the blue box indicates the decidualization period. P = progesterone, E2 = estradiol, GC = glucocorticoid, A = Androgens) (adapted from Maybin and Critchley, 2012).

Extensive research has been conducted on the relationship between GR and GCs and conditions such as heavy menstrual bleeding (HMB), also known as menorrhagia. It has recently been identified that there is a dysregulation of the 11β-HSD enzyme present in the endometrium of patients suffering from HMB. This dysregulation may result in reduced

levels of locally available cortisol being present within the endometrium, leading to impaired angiogenesis, known to be crucial in the regulation of menstruation (Rae et al, 2009). Further research is needed to elucidate the role of GR, its ligands and its consequent signalling pathway during the menstrual cycle in various endometrial and ovarian pathologies, in addition to the normal cycling endometrium.

1.4.1.1 Crosstalk Between Hormone Receptors and the GR Pathway within the Endometrium

In addition to investigating the impact of GCs and the GR signalling pathway on genes which are involved in crucial processes within the endometrium, it is also important to investigate the potential crosstalk between GR and the other steroid hormone receptors such as ER, PR and AR. Extensive crosstalk between GR and ER and PR has been observed elsewhere within the human body to date.

Crosstalk between the steroid hormone receptors is thought to be involved in the control of breast tissue homeostasis and therefore may influence breast cancer development (Vilasco et al, 2011; Kraus, Weis and Katzenellenbogen, 1995; Miranda et al, 2013). Reciprocal inhibitory actions have been revealed between the ER and GR signalling pathways, where GR mRNA levels are higher in ER-negative breast cancer cell lines compared to cell lines which are ER-positive (Vilasco et al, 2011). Additionally, GCs have also been shown to inhibit estrogen regulated ER-positive breast cancer cell proliferation as GCs may inhibit the expression of ER target genes (Karmakar et al, 2013). As estrogen and GCs are both known to influence cell growth and survival, the consequences of the combined effect of both hormones on hormone responsive tissues, such as the breast and endometrium need to be further investigated (Vilasco et al, 2011).

Whole genome micro-array analysis on human endometrial cancer cell lines has revealed that cells treated with both GCs and estradiol results in genes which are both co-regulated by these hormones and many genes that are antagonistically regulated (Whirledge et al, 2013). Furthermore, studies have found that the ER antagonist; ICI, is able to block the anti-inflammatory effects of GCs, however the mechanism of action remains unknown (Cvoro et al, 2011).

In addition to the ER, it is thought that GR may potentially crosstalk with AR. It is known that prostate cancer cells which are resistant to anti-androgen therapy, such as enzalutamide, have increased GR expression. It is thought that GR is able to substitute for AR, and activates a similar array of target genes to maintain the resistant phenotype and allow for

cancer progression (Arora et al, 2013). Therefore, these studies are of great importance in the investigation of the potential mechanisms of crosstalk between AR and GR which may be involved in PCOS development and progression.

As the human endometrium and ovaries are hormone responsive tissues which express the steroid hormone receptors and are therefore targeted by the steroid hormones, a thorough investigation of the potential cross-talk between the GR signalling pathway and other hormone receptors is needed within fertile patients compared to infertile pathologies (Bamberger, Milde-Langosch, Löning and Bamberger, 2001).

1.4.2 The Glucocorticoid Receptor Pathway and Decidualization

It has been previously suggested that the GR signalling pathway and hence the GCs may have a significant role during the critical process of decidualization due to the endometrial expression pattern of GR, where it is specifically expressed within the stromal region throughout the menstrual cycle (Bamberger et al, 2001).

Recent studies on mice have tried to identify the role of GR within the endometrium. Whirledge and colleagues generated a uterine specific GR knockout (KO) mouse, which resulted in a sub-fertile phenotype (S. D. Whirledge et al., 2015). The GR KO mice had defective implantation and stromal cell decidualization (S. D. Whirledge et al., 2015). These initial studies on mice suggest that GR and its signalling pathway play a significant role on fertility status and the establishment and maintenance of pregnancy. Further studies need to be conducted to establish the role of the GR signalling pathway in the human endometrium.

Even though numerous epidemiological and a limited amount of molecular studies suggest that the GR signalling pathway play a role within the normal functioning of the endometrium (Maybin and Critchley, 2012; Kuroda et al, 2013; Bamberger et al, 2001), little research has been undertaken to understand the effect of GC exposure and activation of the GR signalling pathway in infertile pathologies, particularly during the decidual response.

1.4.3 The Glucocorticoid Receptor Pathway and Infertile Pathologies

Research is needed to establish whether the GR signalling pathway has a direct effect on critical processes which take place within the human endometrium. These studies will enable us to investigate whether there is a molecular relationship between stress exposure and the

consequent activation of the GR signalling pathway in infertile pathologies (Campagne, 2006; S. D. Whirledge et al., 2015).

1.4.3.1 GR and PCOS

In addition to the previously mentioned endocrine and reproductive clinical manifestations of PCOS, it has also been proposed that PCOS patients experience a reduced quality of life. Questionnaire and interview studies have found that there is a higher prevalence of psychological disorders such as anxiety, depression, eating disorders, social phobias and poor self-perception in PCOS patients compared to fertile patients and quite shockingly the PCOS patient groups have a higher number of suicide attempts (Barthelmess and Naz, 2014; Conway et al., 2014). The relationship between the hormonal and metabolic profiles and the psychological symptoms seen in PCOS patients remains debatable. However, recent studies have shown that PCOS patients who are clinically suffering from anxiety have higher levels of circulating androgens compared to those experiencing no anxiety and these patients also appear to have an exaggerated stress response (Conway et al., 2014).

As previously mentioned, it is known that women suffering from PCOS have altered cortisol metabolism (Tsilchorozidou et al, 2003). Additionally, it has been found that women suffering from this syndrome have a significantly higher circulating level of cortisol compared to fertile women (Dolfing et al, 2003). Furthermore, it was found that women suffering from PCOS also had significantly higher circulating levels of cortisol metabolites compared to control patients and also display impaired cortisol metabolism (Rodin et al, 1998; Gambineri, 2009).

These studies provide evidence as to why women with PCOS experience high stress levels compared to fertile women and suggests that altered cortisol metabolism resulting in high levels of cortisol and its metabolites being present in PCOS patients may be playing a significant role in the genesis of this syndrome. Due to the high prevalence of psychological disorders in those suffering from PCOS, it is very important to conduct more research to reveal the role of the GR signalling pathway in these patients, particularly the effect of GR on critical processes involved in fertility such as decidualization. And furthermore, whether these patients exhibit an altered GR signalling pathway which may lead to the development and progression of this syndrome (Conway et al., 2014).

1.4.3.2 GR and Endometriosis

Recent research has suggested that the GR signalling pathway may also be playing a role in the development and progression of endometriosis. It is known that women suffering from endometriosis suffer from significant emotional distress which may be attributed to the two major manifestations of the disease, chronic pelvic pain and infertility (Tariverdian et al, 2007). These clinical symptoms of endometriosis can be described as persistent stressors (Tariverdian et al, 2010). Levels of perceived stress in patients suffering from chronic endometriosis are significantly higher than healthy patients, where particularly high levels are observed in patient cohorts which have undergone surgical intervention on numerous occasions and therefore represent the most severe cases of the disease (Lazzeri et al, 2015). Animal models have been used to elucidate that stress can contribute to the development and severity of endometriosis through several mechanisms, including cell recruitment, release of inflammatory mediators and the dysregulation of the HPA axis (Cuevas et al, 2012). It has been hypothesised that elevated stress levels in endometriosis patients may be associated with the numerous comorbidities exhibited in these patients, which include inflammatory conditions and autoimmune disease (Luisi et al, 2015).

It is thought that stress which results in the production of GCs and the consequent activation of the GR signalling pathway may contribute to the high levels of cortisol found locally within the endometriotic tissue and also within the serum, which may have a pathological consequence for endometriosis patients (Monsivais et al, 2012; Lima et al, 2006). Therefore an elucidation of the role stress and the GR signalling pathway in endometriosis patients may provide us with a better understanding of the molecular and cellular mechanisms which underlie the etiology of this complex disease and help us determine whether stress contributes to the pathophysiology of this chronic disease.

1.4.3.3 GR and Unexplained Infertility

Infertility causes major distress in life, where approximately 15-30% of infertile couples are suffering from unexplained infertility (UI). UI is diagnosed only when the results from standard infertility evaluation are normal, such as semen analysis, assessment of ovulation, hysterosalpingogram, tests for ovarian reserve and laparoscopy (Moradan and Ghorbani, 2009).

Questionnaire and interview based studies have found that couples suffering from UI, think that stress or anxiety may be contributing to the outcome of their fertility treatment

(Donarelli et al, 2016). However, the role of stress on infertility remains a controversial topic, despite numerous advances in the field.

An area which has received growing interest in recent years is the role of psychological stress on IVF treatment outcome. It has been found that there are improved pregnancy rates among IVF patients who undergo randomised cognitive behavioural therapy (CBT) compared to those receiving standard care (Domar et al, 2000; Domar et al, 2011). Additionally, women trying to conceive who are treated with anti-depressants and psychotherapy have increased pregnancy rates compared to control groups (Ramezanzadeh et al, 2011). Gourounti and colleagues (2011) found that infertility-specific stress and anxiety is negatively associated with the success rate of pregnancy after IVF treatment in 160 infertile women (Gourounti et al, 2011). Despite this, studies have found no relationship between perceived stress and ovarian response in couples suffering from UI (Donarelli et al, 2016).

Studies have suggested a link between the HPA axis, HPG axis and physical stressors, where it is now widely accepted that stress exposure may inhibit and disrupt a women's menstrual cycle (Lynch et al, 2014). Even though there is growing evidence that stress may exert its deleterious effects on IVF treatment through the well-established HPA axis, research conducted to establish a link between cortisol exposure and IVF outcome has remained inconclusive (Massey et al, 2014). This may be due to the fact that elucidating a role of stress in infertility remains problematic due to the challenges faced when trying to measure stress levels objectively (Lynch et al, 2014).

It is known that cortisol is a classical biomarker of stress; however, Lynch and colleagues were not able to elucidate an association between salivary cortisol levels and fertility status (Lynch et al, 2014). Additionally, there seems to be conflicting evidence as to whether high cortisol levels can lead to a detrimental or a favourable effect on reproduction (Massey et al, 2014). Some studies report that lower cortisol serum levels are associated with clinical pregnancy (An et al, 2013), whereas others suggest that higher cortisol levels within the follicular fluid are associated with higher pregnancy success rates (Thurston et al, 2003). Furthermore, it has been found that dexamethasone treatment, which is a synthetic derivative of cortisol, is able to induce ovulation effectively in infertile patients, but the mechanism of action is unknown (Moradan and Ghorbani, 2009).

Therefore, even though ART is successful in overcoming many fertility problems, more research is needed to elucidate additional factors which may be contributing to infertility, particularly cases which are unexplained. The molecular signalling pathways underlying the

potential relationship between stress exposure, the GR signalling pathway and UI need to be further investigated.

1.4.4 GR and the Development of Cancer

There has been much evidence that stress and hence GCs play a significant role in the development and progression of cancer (Sannes et al, 2013). For example, there has been epidemiological and molecular evidence for an association between exposure to stress and the development of several cancers, including breast cancer (Antonova, Aronson and Mueller, 2011).

Not only is GR extensively expressed within both normal breast tissue and cancerous breast tissue, but it has been found that in the breast cancer cell line; MCF-7, GR is able to exert anti-apoptotic effects and directly affect cellular proliferation (Vilasco et al, 2011). This indicates that stress and the consequent activation of the GR signalling pathway may have a significant role in breast cancer development. However, studies that have investigated the role of stress in the progression and development of breast cancer have provided varied results, where it is hypothesised that the type of stress, whether it be acute or chronic, have independent effects on the risk of developing breast cancer (Vilasco et al, 2011; Volden and Conzen, 2013). Potential crosstalk between GR and the other steroid hormone receptors, such as the ER and PR, within the endometrium has been previously discussed in section 1.4.1.1. This steroid hormone receptor crosstalk is also thought to be involved in the control of breast tissue homeostasis, suggesting that any disruption in this crosstalk may influence breast cancer development (Kraus, Weis and Katzenellenbogen, 1995). For example, reciprocal inhibitory actions between ER and GR signalling have been detected within breast cancer cell lines, where GR mRNA levels are higher in ER-negative breast cancer cells compared to ER-positive cells as previously mentioned (Hall et al, 1990). Therefore, as estrogen and GCs are both known to influence cell growth and survival – the consequences of the combination of both hormones on the hormone responsive breast tissue has to be further explained (Vilasco et al, 2011).

Similarly, the ovaries and the human endometrium are also hormone responsive tissues (Bamberger, Milde-Langosch, Löning and Bamberger, 2001), and it is known that estrogen plays a significant role in the development and progression of cancer within these reproductive organs (Xu, Fang, Liu and Song, 2002; Bender, Beukers and Leslie, 2011). Therefore, clarification of the effect of GCs, estrogen and their corresponding receptors is needed within gynaecological cancers. In addition to breast cancer, GCs and the GR

signalling pathway is also thought to have a role in several gynaecological cancers, including ovarian cancer (OC) and endometrial cancer (EC). However, similar to the fertility studies which have been discussed – studies investigating the role of the GR signalling pathway on the development of gynaecological cancer remain inconclusive. Prospective cohort studies have suggested that stress may be affecting the gonadal synthesis of estrogen which results in altered sensitivity of the uterus towards estrogen stimulation leading to a decreased risk of EC observed among stressed women (Nielsen et al, 2007). In contrast, other studies have found that GCs are regulating cell proliferation within the endometrium and any dysregulation in this regulatory pathway may lead to the development of tumours (Whirledge, Dixon and Cidlowski, 2012).

Furthermore, it is thought that an abnormal cortisol level prior to chemotherapy treatment is associated with decreased survival in patients suffering from OC (Schrepf et al, 2015). However, Woenckhaus and colleagues found that GR expression within OC tissue samples had no effect on prognosis (Woenckhaus et al, 2005).

Therefore, GCs and the GR signalling pathway appear to have a role within oncology, but this remains widely debated suggesting that more research needs to be undertaken. Additionally, there is a growing interest to define the prognostic role of GR expression within cancerous tissues within the body, particularly the breast, ovaries and endometrium. This would allow us to shed light on not only the role of stress and GCs in the development of cancer, but also on the clinical use of GCs and their effect on cancer patients.

1.5 Thesis Aims and Objectives

Given the extensive body of evidence within the literature which implicates stress in propagating infertility, particularly in females suffering from infertile pathologies, this project aims to establish a link between the activation of the GR signalling pathway, endometrial pathology and decidual transformation in both fertile and infertile patients. The principal aim of this thesis is to present novel evidence for the expression of GCs and its nuclear receptor; GR in fertile and infertile patients and to relate these observations to key features of common reproductive endocrinological pathologies namely PCOS, endometriosis and UI. Secondary to this, was to establish the effect of the GR signalling pathway activation in the human endometrium during the process of decidual transformation in fertile and infertile patients.

The work undertaken in this thesis is relevant to the understanding of a potential effect of stress on endometrial infertility.

The objectives of this study were:

- To assess the endometrial expression of GR in both fertile and infertile patients with: PCOS, UI and endometriosis during the proliferative and secretory phase of the menstrual cycle.
- To assess the circulating serum levels of the GCs; cortisol and cortisone in both fertile and infertile women with: PCOS, UI and endometriosis during the menstrual cycle.
- To determine the effect of the GR signalling pathway in the presence and absence of the stress hormones during decidual transformation in the fertile and infertile patients.
- To establish whether the HESC cell line could be used as a suitable *in vitro* model of fertile endometrium to assess the GR signalling pathway during decidua formation.
- To elucidate potential target genes of the GR signalling pathway during decidual transformation and assess their expression levels in response to GR activation in the fertile and infertile endometrium.
- To establish whether crosstalk between the GR signalling pathway and other steroid hormone receptor pathways within the endometrium had a significant effect on decidual transformation in both fertile and infertile patients.

CHAPTER 2

Materials and Methods

2. Materials and Methods

2.1 Patient Recruitment and Samples Obtained for Three Year Study

One hundred and sixteen patients were enrolled in this study. Patient recruitment and consent was carried out at the Gynaecology clinics at Singleton, Neath Port Talbot and Bridgend hospitals (ABMUHB). Ethical approval has been previously obtained from the South Wales Research Ethics Committee (LREC Wales 6) for the duration of this study (three years) (LREC reference: 05/WMW02/103) (see appendices B and D for study protocol, participant information sheets and consent forms). Patients were recruited from gynaecology outpatient clinics and pre-admission clinics. Prior to any data collection under this protocol, a written informed consent was obtained from all patients at the time of recruitment into the study, and was signed by the patient, in accordance with local practice and regulations. Information about the study was explained to the patients and a copy of the informed consent form, signed and dated by the patient was given to the patient. Additionally, confirmation of the patient's informed consent was documented in the patient's medical records prior to any data collection. The study conducted was a non-interventional study; therefore the risks for subjects linked to their participation in the study were limited to breach of confidentiality with regard to personal identifiers or health information. In order to ensure patient confidentiality, biopsies obtained from consenting patients were designated with a specific number to ensure that the anonymity of the patient was upheld. Upon enrolment, the patient was required to provide their name and address, but these were not entered into the clinical study database. The database contains the clinical details and experimental results and was password protected and accessed only by the members of the study team. The chief investigator (Dr Lavinia Margarit) stored the patients' name and identification information separately from other study information and this was locked in secure storage in the investigator's office. The unique study numbers (biopsy number) were the only identifier visible to all other members of the study team.

The inclusion criteria ensured that patients who were fertile and were attending gynaecology clinic and theatre for procedures involving benign disease, such as prolapse for example, patients with PCOS, patients with endometriosis and patients diagnosed with unexplained infertility (UI) were approached and given both verbal and written information.

All patients were pre-menopausal and had not received any exogenous steroid treatment or hormonal therapy for at least 2-3 months prior to surgery and collection of the biopsy. The exclusion criteria of this study ensured that patients who were pregnant or breastfeeding, had a documented evidence of an ongoing infection, those declining to consent, those unable to

give informed consent, women for who English was not their first language or are unable to fully understand the verbal and written information about the study or adolescents under the age of eighteen, were excluded from participating in this study.

Blood samples were also collected from all patients at the time of endometrial biopsy collection for future experiments for the determination of hormone levels in circulation in the various pathologies using Enzyme Linked Immunosorbant Assay's (ELISAs). Ethical approval was in place to collect blood samples obtained for the duration of the study (LREC reference: 12/WA/0289) (see appendices A and C for study protocol, participant information sheets and consent forms).

The primary outcome of this study was to study the blood levels of biomarkers within infertile patients and control patients, and assess the sensitivity and specificity of these markers in predicting infertility status. A secondary outcome of this study were to establish any correlation between the expression of biomarkers in blood and endometrial tissue and to determine the value of the blood biomarker examination along with (or instead of) the endometrial biopsy, as predictive for further pathology or fertility. A further secondary outcome of this study was to assess the levels of biomarkers in combination with the levels of circulating hormones, such as estrogen, progesterone and the gonadotropins, which are routinely measured in clinical practice, to determine the type of infertility and whether the use of these additional biomarkers can improve diagnostics.

2.1.1 Menstrual Cycle Monitoring and Hormone Assay of Patients

The endometrial biopsy samples obtained for this study were from women in a natural menstrual cycle. A regular menstrual cycle was defined as having an intermenstrual interval between 25 and 35 days, with no more than 4 days of variation from one cycle to another. Blood tests were carried on days 2-6 of the patient's menstrual cycle to assess various hormone concentrations to identify the phase of the menstrual cycle. This included investigating the levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), testosterone, prolactin and sex hormone binding globulin (SHBG). Urinary LH was used to document ovulation. A transvaginal sonography (TVS) was also performed to assess ovarian and uterine morphology and to further confirm the phase of the menstrual cycle. The TVS was also used to diagnose PCOS using the Rotterdam criteria and to rule out any other ovarian or uterine pathology. The endometrial biopsies were taken during the proliferative, secretory and menses phase of the menstrual cycle in regular ovulatory cycles. However, the

anovulatory cycles were of variable length and patients had either oligo menorrhea or amenorrhea, where biochemical tests confirmed the absence of ovulation.
As previously mentioned, the patients recruited to this study had not received any exogenous hormonal therapy for at least 2-3 months prior to the procedure.

2.1.2 Clinical Data and Patient Demographics

The control group used for this study contained women with proven fertility and regular menstrual cycles, attending the Obstetrics and Gynaecology department (ABMUHB). These patients were recruited from general gynaecology clinics, having presented for sterilisation with no confirmed diagnosis of endometriosis or Poly Cystic Ovary Syndrome (PCOS).

Infertility was defined as the inability to conceive after 2 years after regular sexual intercourse with a normal male factor. The infertile study group consisted of infertile women with the following pathologies: ovulatory PCOS (ovPCOS), anovulatory PCOS (anPCOS), endometriosis or unexplained infertility (UI).

Endometriosis was diagnosed at the time of laparoscopy that was being performed either for infertility investigations or for the diagnosis of chronic pelvic pain. The diagnosis was further confirmed by visual inspection of the pelvic organs or by histological confirmation of the presence of endometriosis on peritoneal lesions. The endometriosis grades minimal, mild, moderate and severe were included in this study. Pelvic inflammatory disease and tubal blockage were excluded from this study.

PCOS was diagnosed using the Rotterdam criteria (Rotterdam ESHRE/ASRM-Sponsored PCOS Conensus Workshop Group, 2004). The PCOS group were confirmed to have polycystic ovaries (at least 12 discrete follicles of 2-9mm diameter in the ovary and/or increased ovarian volume >10mL) upon transvaginal ultrasound examination, were all nulliparous, had patent fallopian tubes, normal sperm parameters and presented all the clinical and biochemical features of hyperandrogenism. Samples were obtained from patients with ovulatory and anovulatory cycles. Biochemical examinations were used to confirm anovulation in the patients suffering from anovulatory PCOS.

Unexplained infertility was diagnosed as the inability of spontaneous conception in the presence of ovulation, patent tubes (evaluated by laparoscopy and dye test) and normal sperm diagram. For this group of patients, endometriosis was excluded at the time of the laparoscopy. Tubal patency was confirmed by either a Hysterosalpingo-contrast-sonography (HyCoSy) scan or laparoscopy and dye test.

Therefore the study group contained patients with ovPCOS (n = 12 patients), anPCOS (n=22), endometriosis (n = 29 patients) and UI (n = 29 patients), compared with the control group (n = 24 patients). Patients included within the study groups were homogenous in terms of age and BMI when compared to the control patients (Table 2.1).

	Controls (Fertile)	ovPCOS	anPCOS	Endometriosis	UI
No of Patients	Biopsy n=24 Serum n=22	Biopsy n=12 Serum n=14	Biopsy n=22 Serum n=22	Biopsy n=29 Serum n=16	Biopsy n=29 Serum n=14
Age	29.58 ± 4.12	28.72 ± 5.06 (p=0.777)	25.1 ± 4.4 (p=0.267)	28.63 ± 4.55 (p=0.727)	27.1±2.4 (p=0.440)
BMI (kg/m^2)	26.70 ± 4.82	26.65 ± 3.34 (p=0.985)	28.15 ± 2.3 (p=0.591)	27.54 ± 3.08 (p=0.753)	28.6±2.01 (p=0.672)

Table 2.1 Number, Age and BMI of patients from control, PCOS, endometriosis and UI groups recruited for biopsy isolation for Immunohistochemistry and serum Enzyme Linked Immunosorbant Assay (ELISA) analysis. Values for age and BMI are given as mean +/- standard deviation.

2.1.3 Endometrial Biopsy Samples

After informed consent had been obtained, the patients recruited for this study were asked to contact the hospital that was caring for them on day one of their menstrual cycle and were then advised to attend the unit on either day 2- 6 of their cycle. During this visit, blood was taken to estimate their baseline serum levels of FSH, LH, prolactin, testosterone and SHBG and transvaginal sonography was carried out. Subsequently, they were asked to attend on day 21-22 of the cycle when a blood test was used to confirm ovulation. A serum progesterone level of ≥ 30 nm/L confirmed ovulation had taken place. Urinary LH values were also used to document ovulation.

The endometrial biopsies were obtained by curettage concurrent to surgical or diagnostic interventions such as laparoscopy or hysteroscopy. The biopsy samples were divided into two groups, one group for immunohistochemistry (IHC) and the other for *in vitro* studies.

The biopsy samples were used in this study as described below:

1) IHC analysis of Formalin fixed paraffin embedded (FFPE) endometrial biopsy specimens to determine the expression of the glucocorticoid receptor (GR) in the endometrial cells.
2) Quantitative Real-Time PCR (qPCR) and western blot analysis was carried out using RNA and protein extracted from fresh biopsy samples to establish the expression of GR and 11β-hydroxysteroid dehydrogenase 1 and 2 (11β-HSD1 and 2) in fertile, PCOS, endometriosis and UI groups.
3) Endometrial stromal cells (ESCs) were isolated from fresh endometrial biopsy samples and were cultured to form a confluent monolayer of cells. These cells were treated with a combination of treatments, such as cAMP, DHT, P_4, cortisone, cortisol and RU486 for 48h. RNA or protein was extracted and qPCR or western blot analysis was used to determine the expression levels of various proteins and genes respectively. qPCR was used to determine the expression of GR, MR 11β-HSD1/2, dPRL, IGFBP-1, WT1, FKBP4 and FKBP5 at the mRNA level. Furthermore, western blot analysis was used to determine the expression of GR, 11βHSD1/2, PRL, WT1, FKBP5 and the sex steroid hormone receptors at the protein level.

2.2 Immunohistochemistry Analysis of Glucocorticoid Receptor (GR) Expression

2.2.1 Immunohistochemistry Analysis of Patient Samples from Control and Study Groups

Immunohistochemistry (IHC) is a method used to demonstrate the presence and location of proteins within tissue sections. For IHC analysis, control and study samples were fixed in 10 % buffered formaldehyde for 24 h at the time of endometrial biopsy. The fixed samples were embedded in paraffin and cut into sections between 3-4 μM in thickness. Paraffin was removed from the sections (dewaxed) with xylene, incubated through a series of methanol grades, and fixed onto Super frost slides for staining. Routine Haematoxylin and Eosin (H&E) staining was performed on each slide and the sections examined for morphometric dating of the endometrial biopsy according to Noyes criteria modified by Accosta and Lessey which was used to confirm the stage of the menstrual cycle (Acosta et al., 2000; Lessey et al., 2000; Noyes & Haman, 1953; Noyes, Hertig, & Rock, 1975). Samples that had evidence of endometritis, endometrial hyperplasia or endometrial polyps were excluded from this study.

Immunohistochemistry was performed at the Pathology Department, Singleton Hospital using a fully automated IHC/ISH slide-staining instrument, the Ventana Benchmark XT processor (Ventana Biotek Solutions, Tucson, AZ, USA). For the detection of proteins using specific antibodies, barcode labels for each antibody were printed and attached to the slides prior to loading the slides into the instrument. Firstly, the slides were heated in citrate buffer CC1 (Ventana Biotek Solutions Tucson AZ) to 100 ^{O}C for 1 h for antigen retrieval. Next, the slides were incubated with 100 µL of primary antibody at a dilution of 1:50 (Table 2.2).

The time duration and the temperature used for this incubation were optimal for the specific antibody used.

Antibody Name	**Manufacturer: Catalogue Number**	**Target**	**Species**	**Dilution**
Anti-GR	Santa Cruz: sc-8992	GRα and GRβ epitope corresponding to amino acids 121-420	Rabbit polyclonal (H-300)	1/30

Table 2.2 Antibody name, manufacturer: catalogue number and species of origin used for immunohistochemical (IHC) analysis.

Positive immunostaining was detected using Ventana I View DAB detection kit (Ventana Biotek Solutions, Tucson AZ). Staining was visualised by detection of the interaction between Avidin-Biotin peroxidase complex solution and the biotin conjugated secondary antibody. Slides were then counterstained with 100 µL of Haematoxylin stain for 1 min at room temperature, dehydrated, incubated in xylene for 5 min to clear and then left to air-dry and mounted in DPX mountant to be assessed using light microscopy.

There were five groups for IHC protein analysis in this study:

Group 1: Control (Fertile) (n= 24)
Group 2: Ovulatory PCOS (ovPCOS) (n= 12)
Group 3: Anovulatory PCOS (anPCOS) (n= 22)
Group 4: Endometriosis (n= 29)
Group 5: Unexplained Infertility (UI) (n= 29)

These five groups were further divided into the phase of the menstrual cycle identified at the time of endometrial biopsy seen in table 2.3.

Endometrial Pathology	Phase of Menstrual Cycle		
	Proliferative (n=)	Secretory (n=)	Total (n=)
Fertile	10	14	24
ovPCOS	6	6	12
anPCOS	22	0	22
Endometriosis	14	15	29
UI	13	16	29

Table 2.3. Number of patients recruited to IHC study and number of patients in each phase of the menstrual cycle.

2.2.2 Scoring of IHC Slides

Fixed slides were visualised using a Zeiss light microscope at x40 objective. Immunoreactive signal was detected by the presence of brown staining. Each slide was consequently scored by three independent observers for both the intensity and the distribution of the protein in both the glandular and luminal epithelium and within the stroma. The first two observers (Dr Deyarina Gonzalez and I) scored all slides, whilst the third and final observer (Dr Lavinia Margarit) scored ten slides from each subgroup of patients (i.e. fertile, ovPCOS, anPCOS, endometriosis and UI). The cellular location of the protein staining was also noted i.e. whether it was present in the nucleus or cytoplasm. Each scorer used an immunohistochemical scoring system which has been previously described by (Lai et al., 2005). Positive red-brown stain was scored for intensity and for the distribution of the staining, which is known as the H-score. Intensity was scored from 0-4, where 0 signifies the absence of a stain and 4 signifies the presence of a very strong stain. The distribution of stain throughout the tissue was scored as follows, 0 = absent, 1 = less than 30 %, 2 = 30-60 %, 3 = more than 60 % and finally 4 = 100 %. Positive and negative controls were also scored, where the positive control was tonsil and the negative control was endometrial tissue incubated with mouse or rabbit IgG, and adjacent sections were cut and stained in parallel using identical procedures. Scoring of the controls allowed for more accurate scoring of the tissue samples being investigated. The observers were also blinded to the patient's diagnosis and demographics prior to scoring the slides.

2.2.3 Statistical Analysis of IHC Scores

To determine the optimal sample size for this study and to ensure that the study was able to distinguish an actual effect from one of chance, the statistical power of the study was investigated. Sample sizes for each group under investigation were chosen to ensure that the recommended minimum value for the power of the statistical tests being utilised in this study to be 0.8. The power analysis and sample size determination for this study was carried out using NCSS PASS software (NCSS LLC, Kaysville, Utah).

The distribution of the data was assessed for normality using the Kolmogorov-Smirnov test, where non-normally distributed data was analysed using the non-parametric Kruskal Wallis test, which was followed by a Mann-Whitney U test to determine the statistical significance of the data. After the test of normality, it was deemed that all IHC data was not normally distributed, and therefore was analysed using this method. Statistical analysis was performed using SPSS software – version 10.0 (SPSS, Chicago, IL). A p value of less than or equal to 0.05 ($p \leq 0.05$) was considered to be statistically significant and was denoted by the presence of one star (*), whereas a p value of less than or equal to 0.01 ($p \leq 0.01$) was denoted by the presence of two stars (**), and finally a p value of less than or equal to 0.001 ($p \leq 0.001$) was denoted by the presence of three stars (***).

2.3 Cell Culture

2.3.1 Introduction to Endometrial Cell Line Used

Only one endometrial cell line was used during this study, which represented the stromal compartment of the endometrium. This cell line is known as the human endometrial stromal cell (HESC) cell line (Krikun et al., 2004). HESCs were cultured from a woman of reproductive age, where the cells were obtained during a hysterectomy for a benign condition. The cells were then immortalised using human telomerase reverse transcription (hTERT), hTERT was used to add TTAGGG repeats to telomeres to compensate for their shortening. The HESC cell line contains no chromosomal, structural or numerical abnormalities and is shown to display similar outcomes to primary, un-passaged stromal cells after treatment with ovarian hormones. HESCs are karyotypically, morphologically and phenotypically comparable to primary cells, these cells respond to hormone stimulation, and are commonly used as valuable tool in endometrium homeostasis and female reproductive studies (Krikun et al., 2004). This HESC cell line was purchased from Applied Biological Materials (abm, Canada).

2.3.2 Culture of Cell Lines

The HESC cell line previously described has adherent properties, and was therefore cultured in plastic culture vessels (Falcon T25, T75 T125) and maintained in Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM/F-12) in a 1:1 ratio with GlutaMAX^{TM} (2.5 mM), supplemented with 10% foetal bovine serum (FBS), 1 mM sodium pyruvate, 1 mM sodium bicarbonate (1.5 g/L – stock 7.5 %) and 1 % penicillin/streptomycin. Cells were incubated and maintained at 37 ^{o}C in a humidified 5 % CO_2 atmosphere (Nuaire, UK). The cells were sub-cultured according to the guidelines set by the distributor, where the HESC cells were split at a sub-cultivation ratio of 1:3 at 70-80 % confluence.

In order to sub-culture the cells, cells were initially washed in calcium and magnesium free phosphate buffered saline (PBS) (Gibco, UK) in order to maintain osmotic balance, pH and also to promote cell detachment as the PBS removes any remaining DMEM/F12 media residue from the cell monolayer. Subsequently, after the wash step the cells were detached from the plastic culture vessel by incubating the cell monolayer with 0.25 % trypsin 1 mM Ethylenediaminetetraacetic acid (EDTA) (pH 8.0) in Hanks' Balanced Salt Solution (HBSS) containing phenol red and in the absence of $CaCl_2$, $MgCl_2$ and $MgSO_4$ (Gibco Invitrogen, Cat No: 25200-056). The cells were incubated with trypsin for approximately 5 min at 37^{O}C. Detachment was confirmed using inverted light microscopy (Zeiss). Once detachment of the cell monolayer was confirmed, 10 mL of fully supplemented DMEM/F-12 media was added to the cell suspension in order to neutralise the trypsin and prevent any further enzymatic activity that could result in damage to the cells. The supplemented DMEM/F12 media contains calcium, magnesium and bovine pancreatic trypsin inhibitor which blocks the anti-adherent action of the trypsin enzyme (Borjigin & Nathans, 1993). The suspended cells were transferred to a 15 mL centrifuge tube (Eppendorf, UK) and consequently centrifuged for 5 min at < 1200g. Excess media was poured out and the remaining cell pellet was re-suspended in 10 mL DMEM/F-12 media. The appropriate ratio of cells was taken from the cell suspension and incubated in the correct culture vessel in fresh media at 37 ^{O}C in a humidified CO_2 atmosphere. All materials in this section were obtained from Gibco Invitrogen unless otherwise stated.

2.4 Primary Cell Culture

2.4.1 Isolation of Primary Endometrial Stromal Cells from Biopsy Samples

All primary cell culture was performed in class II safety cabinet and all surfaces were cleaned with 70% ethanol and the cabinet was left running for a minimum of 30 min prior to work being started. The biopsy samples were collected in DMEM containing an antibiotic-antimycotic solution. The tissue was washed twice in DMEM solution and also washed in PBS. The tissue was finely diced using a scalpel and enzymatically digested using collagenase (Sigma, UK) for 1h at 37^{o}C. The digested tissue was collected in a 15mL centrifuge tube and centrifuged at 400 g for 4 min.

The pellet obtained was re-suspended in medium, which contained DMEM/F-12, 10% dextran-coated charcoal stripped FBS, 1 mM sodium pyruvate, 1 mM sodium bicarbonate (1.5 g/L – stock 7.5 %) and 1 % penicillin/streptomycin. The cells were plated in a small flask and grown to confluence in the maintenance media. After approximately 24 – 48 h, the medium, which contained the non-attached epithelial cells and cell debris, was decanted and the media was replaced with fresh media. This protocol results in a ≥ 95 % pure endometrial stromal cell (ESC) culture (Dimitriadis, Robb, & Salamonsen, 2002). The proliferating cells were cultured and maintained in media until confluence following the same protocols described in Culture of Cell Lines section 2.3.2.

2.4.2 Plating Cell Lines or Primary ESCs for RNA, Protein or Chromatin Extraction

In preparation for the *in vitro* incubation with steroid hormones, either the HESC cell line or primary ESCs were plated into 6 well plates (Greiner Cell Star, Cat No: 662160) for RNA and protein extraction or T75 flasks for chromatin extraction. For RNA and protein extraction, the cells were processed and cultured as described in the Culture of Cell Lines (Section 2.3.2) and Isolation of Primary Endometrial Stromal Cells (ESCs) from Biopsy Samples (Section 2.4.2). However, when the cells were re-suspended in 10 mL of fully supplemented media, the cells were counted using the TC10 automated cell counter (Bio Rad, UK). In order to accurately count the cells, 50 µL of cell suspension was diluted in a 1:1 ratio with trypan blue, where trypan blue is able to enter the nucleus of dead cells which allows these cells to be omitted from the cell count. 10 µL of the cell suspension containing trypan blue was pipetted onto a dual chamber counting slide (Bio Rad, UK). This counting slide was placed into the cell counter, which automatically estimates the amount of cells present/mL, excluding the cells which have trypan blue within their nucleus signifying that these are not viable cells. Cells were counted in duplicate using this method and an average

value for cell number was calculated. The amount of cells was then adjusted by adding fully supplemented DMEM/F-12 media. For protein and RNA analysis, 2 x 10^5 of HESCs or 6 x 10^5 of primary ESCs were added to 6-well plates (Greiner Cell Star, Cat No: 662160). For chromatin analysis, 1.2 x 10^6 of HESCs were added to a T75 flask. A high concentration of cells was necessary for the HESC cell line and primary ESCs, as they have a reduced growth rate compared to other cell lines. After seeding, the 6 well plates were maintained in a 5 % CO_2 humidified atmosphere at 37 ^{O}C for 24 h or until 70 % confluent.

After the 24 h incubation with fully supplemented media, the cell monolayer was washed with PBS and media was replaced with DMEM/F-12 media supplemented with 10 % dextran coated, charcoal treated FBS (Gibco, UK), 1.5 mM glutamine, 1 mM sodium bicarbonate, 1 mM sodium pyruvate and 1% penicillin/streptomycin, which is the stripped media. The charcoal stripped FBS was prepared by adding 1 g of charcoal (Dextran coated charcoal, Sigma, UK) to 500 mL of FBS and inverted to mix. The FBS was then filtered through a stericup (Millipore, UK) using a vacuum pump system in a lamina flow hood. After addition of this media, plates were incubated for a further 24 h prior to treatment. The addition of charcoal stripped media for a minimum of 24 h before treatment allows for the removal of any trace steroid or larger molecular weight proteins within the media, which may affect the efficiency of the cell treatment.

After the second incubation period with stripped media, the media was replaced with fresh stripped media and when the cell monolayer was 80 – 90 % confluent, cell treatments were added for 48 h. An established *in vitro* model of decidualization which has been widely published in previous research has been used, which is based on the stimulation of Protein Kinase A (PKA) by cyclic adenosine monophosphate (cAMP) or its analogues to initiate decidualization of ESCs (Dunn, Kelly, & Critchley, 2003).

The HESC cell line and the primary ESCs were treated with the following treatments for 48h (either alone or in combination); cortisol (denoted as compound F or F) cortisone (denoted as compound E or E), Progesterone (P_4), 8-Bromoadenosine 3', 5' Cyclic Monophosphate (cAMP) and 5α-Androstan-17β-ol-3-one (DHT), mifepristone (RU486) or untreated (Ctrl). After 48 h of treatment, RNA, protein, chromatin or cell culture media were extracted from the cell monolayer and used for downstream analysis.

2.4.3 Preparation of Cell Treatment

Hormone treatment regimens were chosen at concentrations and time points, which closely mimic the menstrual cycle *in vitro* in a cell line model and primary ESCs.

Confluent endometrial stromal cells monolayers (HESC or primary cells isolated from biopsies) were incubated with treatments for 48h and protein and RNA was extracted after the incubation time to analyse expression levels of GR and its targets in these cells. Treatments were as follows:

Cortisone (E):

Cortisone (E) was prepared by adding 0.036 g of powdered E (Cat: C2755 ≥ 98%, Sigma, UK) to 10 mL of absolute ethanol (100%) (Fisher Scientific, UK) and filter sterilised to produce a stock concentration of 10 mM. This solution was diluted 1:10 to produce a second solution at a concentration of 1 mM. This was further serially diluted 1:10 to produce the working stock concentration of 0.1 mM. When required, 1 µL of this working stock was added per 1 mL of stripped media, which resulted in a final treatment concentration of 0.1 µM (Kuroda et al., 2013).

Cortisol (F):

Cortisol (F) was prepared by adding 0.036 g of powdered F (Cat: H4001 ≥ 98 %, Sigma, UK) to 10 mL of absolute ethanol (100 %) and filter sterilised to produce a stock concentration of 10 mM. This solution was diluted 1:10 to produce the working stock solution at a concentration of 1 mM. When required, 1 µL of this working stock was added per 1 mL of stripped media, which resulted in a final treatment concentration of 1 µM.

8-Bromoadenosine 3', 5' – Cyclic Monophosphate (cAMP):

cAMP was prepared by adding 4.76 mL of distilled water directly to 100 mg of cAMP powder (Cat: B7880, Sigma, UK) and filter sterilised to give a working stock concentration of 50 mM. When required, 10 µL of this working stock was added per 1 mL of stripped media to give a final concentration of 0.5 mM.

Progesterone (P_4):

P_4 was prepared by adding 0.00314g of powdered P_4 (Cat: PO130, Sigma, UK) to 1 mL of absolute ethanol (100 %) (Fisher Scientific, UK) and filter sterilised to give a concentration of 10mM. This was diluted 1:10 to give a working stock concentration of 1mM. When required, 1µL of this working stock was added per 1mL of stripped media, which resulted in a final treatment concentration of 1µM.

5α- Androstan-17β-ol-3-one (DHT):

DHT was prepared by adding 0.029 g of powdered DHT (Cat: A8380, Sigma, UK) to 1 mL of absolute ethanol (100 %) (Fisher Scientific, UK) and filter sterilised to give a stock concentration of 100 mM. This solution was diluted 1:100 to produce a second solution at a concentration of 1 mM. This was further serially diluted 1:100 to produce a working stock concentration of 0.1 mM. When required, 1 µL of this working stock was added per 1 mL of stripped media, which resulted in a final treatment concentration of 10 nM.

RU-486 (or Mifepristone):

RU486 was prepared by adding 0.043 g of powdered RU486 (Cat: M8046, Sigma, UK) to 10mL of absolute ethanol (100 %) (Fisher Scientific, UK) and filter sterilised to give a stock concentration of 10 mM. This solution was diluted 1:10 to produce the working stock concentration of 1 mM. When required, 1 µL of this working stock was added per 1 mL of stripped media, which resulted in a final treatment concentration of 1 µM. in addition, cells were also incubated with this treatment for 30 min, 1h, 4hrs, 12hrs, 24hrs and 48hrs.

2.5 Western Blot Analysis

HESC cells or primary endometrial stromal cells (ESCs) were grown to confluency in a six well plate as described in Plating Cell Lines or Primary ESCs for RNA, Protein or Chromatin Extraction (Section 2.4.3.). Cells were scraped directly in 50 µL of Radio-Immunoprecipitation Assay (RIPA) lysis and extraction buffer (Cat: R0278, Sigma, UK). This buffer enables rapid, efficient cell lysis and solubilisation of proteins from adherent cultured cells. The RIPA buffer also minimises non-specific protein-binding interactions to keep background low. This extraction buffer consists of 50 mM Tris-HCl (pH 8), with 150 mM sodium chloride, 1.0 % Igepal CA-630 (NP-40), 0.5 % sodium deoxycholate and 0.1 % sodium dodecyl sulphate (SDS). Protease inhibitor cocktail (Cat: P8340, Sigma, UK) and phosphatase inhibitor cocktail for both Serine/Threonine phosphatases and L-Isozymes of alkaline phosphatases (Cat: P2850, Sigma, UK) and for Tyrosine protein phosphatases, acid and alkaline phosphatase (Cat: P5726, Sigma, UK) were added to the RIPA buffer at a 1:100 dilution.

2.5.1 Protein Quantification for Western Blot Analysis

Prior to western blot analysis, the protein was quantified using the *DC*™ Protein Assay (Bio Rad, UK) following manufacturer's guidelines. This is a colorimetric assay and is similar to the well-documented Lowry assay. Firstly, the working reagent A' was prepared by adding 20 µL of reagent S per 1 mL of reagent A. 5 µL of samples were then pipetted into a clean, dry microtiter plate and 25 µL of reagent A' was added to each well. 200 µL of reagent B was consequently added to each well. The plate was gently agitated to ensure reagents were fully mixed and if any bubbles were formed, they were popped with a clean, dry pipette tip, ensuring to avoid any cross-contamination of the sample. After approximately 15 min, the absorbance was read at an absorbance of λ750 nm using the FLUOR star OPTIMA plate reader (Labtech, UK). A protein standard curve was generated using known concentrations of bovine serum albumin (BSA) (Fisher Scientific, UK) diluted in extraction buffer to give a range of concentrations from 0.5 mg/mL to 10 mg/mL and extraction buffer alone was used as the blank. The concentration of protein present in the samples was then calculated using the equation; $y = mx + c$, which is the equation of the straight line from the known BSA standards and inputting the values obtained for the samples with unknown concentration into this equation. The quantified protein was consequently adjusted to 30 µg for western blot analysis.

2.5.2 Protein Separation by Gel Electrophoresis

The separation of proteins was successfully achieved using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). 10 % polyacrylamide gels were prepared for SDS-PAGE using the components listed in table 2.4. These values refer to the volumes needed to prepare two polyacrylamide gels.

Firstly, the resolving gel was made using the appropriate volumes of reagent. The polymerising catalysts, 10 % ammonium persulfate (APS) was added last to polymerise the gel matrix in a reaction with the tetramethylethylenediamine (TEMED). The resolving gel was added and set between 1.5 mm separated glass plates (Bio Rad, UK). 2-propanol (Cat: 190764, Sigma, UK) was pipetted on top of the gel to create a flat interface for the application of the stacking gel and to prevent dehydration of the resolving whilst it was setting. After the resolving gel had set, the 2-propanol was removed using distilled MilliQ water (Millipore, UK) and blotting paper (Bio Rad, UK). The stacking gel was then added to the top of the resolving gel and a 1.5 mm 10 well comb was inserted into the solution until the stacking gel was fully set.

Gel Reagent	Resolving Gel (10 %)	Stacking Gel (4 %)
MilliQ Distilled Water (Millipore, UK)	6 mL	3 mL
30% Acrylamide/Bis Solution 37.5:1 (Bio Rad, UK)	5 mL	650 µL
1.5M Tris (pH 8.8)	3.75 mL	-
1.0M Tris (pH 6.8)	-	1.25 mL
10% SDS (Fisher Scientific, UK)	150 µL	50 µL
10% APS (Sigma, UK)	75 µL	25 µL
TEMED (Sigma, UK)	15 µL	5 µL

Table 2.4. Reagents and volumes required for the production of the stacking and resolving gels to create the 10 % polyacrylamide gels required for gel electrophoresis.

To prepare the protein samples for gel electrophoresis, 30 µg of the protein samples were added in a 1:1 ratio with 2x concentrated Laemmli buffer (Cat no: 1610737, Bio Rad, UK). The Laemmli buffer contains glycerol, which allows the protein samples to sink to the bottom of the well and it also contains bromophenol blue, which is a tracking dye and migrates through the polyacrylamide gel first and allows us to visually inspect how far the proteins have travelled through the gel. Additionally, Laemmli buffer also contains SDS detergent, which enables the protein to be fully denatured, removes the higher order structures and ensures that the protein is uniformly negatively charged, therefore allowing the protein to migrate from the negative to positive electrode during electrophoresis. The Precision Plus Protein^{TM} Dual Colour Standard (Cat: 1610374, Bio Rad, UK), which is a colour stained molecular weight marker used for SDS-PAGE gels and western blots, was also diluted in Laemmli buffer at the same ratio as previously mentioned, which guarantees that the molecular weight marker will travel at the same rate as the samples, allowing for accurate estimation of molecular weight.

After the Laemmli buffer was added, all samples were incubated at 95 ^{o}C for 10 min in the T100^{TM} Thermal cycler (Bio Rad, UK), to denature the protein. Equal amounts of protein (30 µg) were loaded into the required wells and 4 µL of the molecular weight marker (Bio Rad, UK) was added to the appropriate wells of the gel. Samples were run on the 10 % SDS-PAGE gel (as previously described) at 120 v for approximately 70 min, or until the dye-front had reached the base of the gel, in ice-cold running buffer. Running buffer (pH 8.3) for gel electrophoresis was prepared by adding 30.3 g of Tris Base, 144.1 g of glycine, 50 mL 20 %

(w/v) SDS, and was made up to 1 L using distilled water. pH was tested and 100 mL of this solution was added to 900 mL of distilled water to make up the final running buffer.

2.5.3 Protein Transfer to Membrane

Separated proteins were consequently transferred to an Immun-Blot® Polyvinylidene difluoride (PVDF) membrane with 0.2 µM pore size and binding capacity of 150-160 µg/cm^2 (Cat: 1620177, Bio Rad, UK), which was cut slightly bigger than the electrophoresis gel. The PVDF membrane was activated for 15sec in absolute methanol (Fisher Scientific, UK), transferred to distilled water (Millipore, UK) for 5min and finally allowed to equilibrate in transfer buffer for 10 min prior to transfer. Transfer buffer was prepared using the components listed in table 2.5. The activated membrane was loaded into a Bio Rad cassette directly against the SDS gel, and filter paper and sponges were added in a specific order to envelope the gel and membrane (figure 2.1).

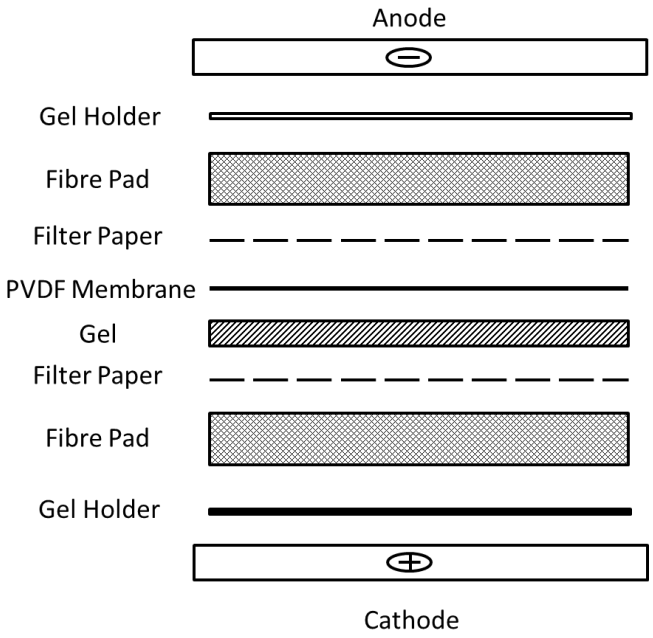


Figure 2.1 Layout of components for transfer to cassette, which is used for protein transfer from an electrophoresis gel to the PVDF membrane.

The fibre pads and filter paper were also equilibrated in the transfer buffer and when assembling the transfer cassette, it was ensured that there were no air bubbles present between the PVDF membrane and the gel using a roller. The separated proteins were transferred onto the activated PVDF membrane in ice-cold transfer buffer for approximately 80 min at 400 mA at 4 ^{o}C, where the appearance of the molecular weight marker on the PVDF membrane was used to confirm that successful protein transfer had taken place.

Reagent	Component	Quantity
Transfer Buffer	Tris-Base	3.03 g
	Glycine	14.41 g
	20% SDS (w/v)	5 mL
	Methanol	200 mL
	dH_2O	800 mL

Table 2.5. Reagents and quantities required for the production of the transfer buffer required for gel electrophoresis.

2.5.4 Protein Detection

Following successful transfer of the gel to the PVDF membrane, the membrane was removed from the cassette and blocked for 1 h at room temperature on a see saw rocker at 30 rpm and a 7 ^{o}C angle of tilt (Bibby Scientific, UK) in blocking buffer containing 5 % BSA in 1x Tris-buffered saline and Tween 20 (TBS/T) (Bio Rad, UK), (Table 2.6). 1x TBS/T was prepared from the stock solution of 10x TBS, which was adjusted to pH 7.6 using Hydrochloric acid (HCl) (Sigma, UK)

Reagent Name	Component	Quantity
10x Tris-buffered saline (TBS) (pH 7.6)	Tris Base	24.2 g
	Sodium Chloride (NaCl)	80.1 g
	dH_2O	1000 mL
1x Tris-buffered saline/ Tween (TBS/T)	10x TBS (pH 7.6)	100 mL
	Tween20	1 mL
	dH2O	900 mL

Table 2.6 Reagents and quantities required for the production of the Tris-buffered saline buffer (10x) and the consequent TBS/T (1x) wash buffer required for gel electrophoresis.

After blocking, the membrane was washed three times for 10 min in 1x TBS/T and the primary antibody was added to blocking buffer, added to the membrane, and consequently incubated overnight at 4 ^{o}C on the see saw rocker at 30 rpm and a 7 ^{o}C angle of tilt. Antibodies were either added to target the protein of interest or for the housekeeping protein used for normalisation of western blot experiments i.e. β-actin. The antibodies and the concentrations used for western blot analysis are described in Table 2.7. All antibodies used for western blot analysis in this study were from commercial sources and all are validated for the use in western blots (please see appendix E for full size immunoblots for each antibody used in this study alongside the molecular weight ladder (Precision Plus Protein^{TM} Dual

Colour Standard, BioRad), as a means of validating antibody quality and validating the observed molecular weight of the bands obtained compared to the expected molecular weight).

	Antibody Name	Manufacturer: Catalogue Number	Species	Dilution (Antibody: Blocking Buffer)	Estimated Molecular Weight (kDa)
Primary Antibodies	Vinculin (EPR8185)	Abcam: ab129002	Rabbit Monoclonal	1:5000	124
	GRα/β (H-300)	Santa Cruz: sc-8992	Rabbit Polyclonal	1:500	95/90
	AR (AR 441)	Abcam: ab9474	Mouse Monoclonal	1:200	110/75
	PR A/B (F-4)	Santa Cruz: sc-166169	Mouse Monoclonal	1:500	81/116
	ERα (HC-20)	Santa Cruz: sc-543	Rabbit Polyclonal	1:500	66
	11β-HSD1	Abcam: ab83522	Rabbit Polyclonal	1:250	32
	11β-HSD2 (C-9)	Santa Cruz: sc-365529	Mouse Monoclonal	1:750	40
	Prolactin (A-7)	Santa Cruz: sc-46698	Mouse Monoclonal	1:500	27
	WT1 (H-290)	Santa Cruz: sc-68880	Rabbit Polyclonal	1:250	52
	FKBP51 (D-4)	Santa Cruz: sc-271547	Mouse Monoclonal	1:500	51
Secondary Antibodies	Anti-Rabbit IgG- HRP	GE Life Sciences: NA934	-	1:1000	-
	Anti-Mouse IgG- HRP	GE Life Sciences: NA931	-	1:1000	-

Table 2.7 The antibodies used for western blot analysis, including manufacturer, catalogue number, species of origin, dilution of antibody used and expected molecular weight (kDa).

After incubation with the primary antibody, the membrane was again washed three times for 10 min in 1x TBS/T and consequently incubated with either anti-rabbit or anti-mouse IgG-

horseradish peroxidase (HRP) conjugated secondary antibody (Table 2.7) (GE Life Sciences, UK) diluted in block buffer, for 1 h at room temperature on a see saw rocker at 30 rpm and a 7 ^{o}C angle of tilt. A final three washes were then carried out to reduce non-specific binding of the antibody. The membrane was finally incubated for approximately 1 min at room temperature with the enhanced chemiluminescence (ECL) stain (Bio Rad, UK). The secondary antibody used for western blot analysis is coupled to a HRP label as previously mentioned, and the ECL stain contains the substrate luminol, which can be oxidised by HRP to produce light. The light emitted from this reaction allows the visualization of a protein band using the Chemidoc^{TM} MP Imaging system (Bio Rad, UK).

2.5.5 Western Blot Data Analysis

The fluorescent bands obtained after visualizing the protein bands using the Chemidoc^{TM} MP Imaging system were subsequently analysed using the Image Lab Software 3.0.1 (Bio Rad, UK). The volume rectangle tool was used to draw around the protein bands of interest and also the background, and the band density was quantified by calculating the signal intensity/area (mm^2). The volume rectangle tool enabled the signal of each band to be quantified in equal defined boundaries. The band density of the housekeeping protein (reference protein), vinculin, was also quantified using this software. This value for vinculin was used as a reference value to normalise the band density of the protein of interest i.e. GR. The values obtained for the band densities were then plotted on a graph using Microsoft Excel.

2.6 Real Time Polymerase Chain Reaction (qPCR)

2.6.1 Isolation and Quantification of Ribonucleic Acid

For ribose nucleic acid (RNA) analysis, the RNeasy kit (Qiagen, UK) was used to isolate RNA, where all components discussed were supplied with the kit unless otherwise stated. The manufacturer's protocol for 'Total RNA isolation from animal tissues' was followed. Briefly, after the 48 h of treatment, the cell monolayer was washed with PBS and the appropriate volume of RLT lysis buffer (350 µL for a 6 well plate) was added to the confluent monolayer. RLT lysis buffer contains a high concentration of guanidine isothyiocyanate, which supports the binding of RNA to the silica membrane during RNA extraction. After RLT lysis buffer was added, the cell monolayer was scraped using cell

scrapers (Greiner Bio-One, UK) and the cell lysate was pipetted directly into the Qiashredder spin columns for homogenisation to break open the cell membranes. The homogenised lysate was subsequently combined in a 1:1 ratio with 70 % ethanol (Sigma, UK) and this mixture was transferred to the RNeasy spin column. The RNeasy spin columns contain a membrane, which is able to bind total RNA. Additionally, a DNase I digestion kit was carried out on column to eliminate the unwanted genomic DNA, which has also bound the membrane along with the RNA using the Ribonuclease-free DNase I kit (Qiagen, UK). For DNase I digestion, 10 µL of DNase I was added to 70 µL of RDD buffer and the solution was added directly to the RNeasy spin column membrane and incubated on the benchtop for 15 min.

After a series of washes in RPE buffer, which is a mild washing buffer mainly used to remove any traces of salts, which are still on the column due to the buffers used earlier in the protocol, the RNA was eluted in 30 µL of RNase free water. Centrifugation steps were performed between each step and all centrifugation was carried out using the Eppendorf™ 5424 micro centrifuge (Eppendorf). Eluted RNA was quantified using a spectrophotometer (Nanodrop 2000c – Nucleic acid programme, Thermo Scientific).

2.6.2 Reverse Transcription Synthesis of cDNA from RNA

RNA at a concentration of 100 ng/µL was reverse transcribed using the high capacity cDNA reverse transcription kit (Applied Biosciences, UK) following the manufacturers guidelines. Firstly, RNA was adjusted to the desired concentration of 100 ng/µL in 30 µL of RNase-free water (Qiagen, UK). 10 µL of this solution was added to a master mix of the components from the reverse transcription kit in the volumes described in Table 2.8 to avoid small volume pipetting errors.

10 µL of the master mix and 10 µL of RNA were mixed gently and centrifuged briefly to spin down the contents and eliminate any air bubbles. Furthermore, 2 µL of the original RNA sample was added to 28 µL of RNase free water and was used as a negative PCR control (un-transcribed template).

The samples were then run on the T100TM Thermal Cycler (Bio Rad, UK) using the following programme. Samples were first heated to 25°C for 10 min, and then further heated to 37°C for 120 min, which allows for cDNA synthesis, this was followed by 85°C for 5 min, which was required to inactivate the reverse transcriptase enzyme and hence stop the reaction. Samples were held at 4 ^{o}C until the samples were removed from the machine.

Component	Volume (µL)
10x RT Buffer	2
25x dNTP Mix (100mM)	0.8
10x RT Random Primers	2
MultiScribe™ Reverse Transcriptase	1
RNase Inhibitor	1
Nuclease-free H_2O	3.2
Total Per Reaction	10

Table 2.8 Volumes of components per reaction needed for the reverse transcription of RNA to cDNA.

The cDNA samples were either stored at -20°C or -80°C for long-term storage. Serial dilutions of the cDNA were made at 1:5, 1:10, 1:100 and 1:1000 (cDNA: nuclease free water) and was used to generate standard curves for PCR cycle quantification. The 1:10 dilution was used to assess target gene expression during qPCR analysis.

2.6.3 Primers used in Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Quantitative real-time PCR (qPCR) was used to amplify cDNA against specific primer pairs. The primers used for qPCR experiments are described in table 2.9.

Primer sets were either designed using Beacon Design software (Beacon Design 7.0, Premier Biosoft, USA) (Table 2.9) or primer set sequences were obtained from published papers (referenced below), evaluated using Beacon Design software and were subsequently ordered through Sigma Aldrich. The primers were designed to obtain a qPCR product between 75 - 150 base pairs (bps), and were ordered with a synthesis scale of 0.025 µmol and purification desalt. The primers were ordered dry in a powder constitution and were diluted to make a 100 µM stock upon arrival. When the primers were needed for qPCR experiments, the primers were diluted with nuclease free water to make a working stock solution of 4 µM.

Gene	Primer	Primer Sequence (5'-3')	(Ta)/°C
Ribosomal Protein 60s L-19 (RPL-19)	Sense	CCTGTACGGTCCATTC	56.2
	Anti-Sense	AATCCTCATTCTCCTCATCC	
Decidual Prolactin (dPRL)	Sense	CCACTACATCCATAACCTCTCC	58
	Anti-Sense	GGGCTTGCTCCTTCTCTTC	
11β-Hydroxysteroid Dehydrogenase Type 1 (11β-HSD1) *	Sense	AGCAAGTTTGCTTTGGATGG	56.4
	Anti-Sense	AGAGCTCCCCCTTTGATGAT	
11β-Hydroxysteroid Dehydrogenase Type 2 (11β-HSD2) +	Sense	GGCCAAGGTTTCCCAGTGA	56
	Anti-Sense	GTTGTGCCAGGAGGGGTGTTT	
Glucocorticoid Receptor α (GRα) *	Sense	CCCTACCCTGGTGTCACTGT	56.7
	Anti-Sense	GGTCATTTGGTCATCCAGGT	
FK506 Binding Protein 4 (FKBP4) #	Sense	AGATGACAGCCGAGGAGATG	55.4
	Anti-Sense	AATTTGTCCTTGCGATCCAG	
FK506 Binding Protein 5 (FKBP5) #	Sense	AAAAGGCCAAGGAGCACAAC	55
	Anti-Sense	TTGAGGAGGGGCCGAGTTC	
Wilms Tumour 1 (WT1)	Sense	CTATTCGCAATCAGGGTTACAG	53
	Anti-Sense	CATGCTTGAATGAGTGGTTGG	
Insulin Like Growth Factor Binding Protein 1 (IGFBP-1) =	Sense	CGAAGGTCTCCATGTCACCA	58.4
	Anti-Sense	TGTCTCCTGTGCCTTGGCTAAAC	
Mineralocorticoid Receptor (MR) *	Sense	GGCACTCGCTGGCCTGGATG	54.3
	Anti-Sense	GTCTCCATCGCTGCCTCGGC	

Table 2.9 Gene specific primers used for real-time PCR (qPCR) to quantify mRNA expression levels. The specific sequences (5'-3') of the gene specific primers and their optimum annealing temperature (Ta) used during the qPCR experiments are shown. Primers marked with '*' were obtained from Kuroda et al, 2013, Primers marked with '+' were obtained from Rae et al, 2009, primers marked with '#' were obtained from Yang et al, 2012 and finally, primers marked with '=' were obtained from Tamura et al, 2012.

2.6.4 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Quantitative Real-Time Polymerase Chain Reaction (qPCR) was used to amplify cDNA against the previously described gene specific primers (Table 2.9), in order to obtain an amplicon of approximately 75-150 bps in length for each target gene under investigation. All results obtained were normalised against an internal reference gene, Ribosomal Protein 60s L-19 (RPL-19) which has an amplicon of 144 bps. The primers were also run against the RNA template and genomic DNA, which are used as the negative and positive control respectively. The amplification reactions were prepared in 10 µL by the addition of the following components shown in Table 2.10.

Component	Volume (µL)
SYBRgreen – SsoFast™ EvaGreen Supermix (Cat No: 172-5203, Bio Rad, UK)	5
Forward and Reverse Primer Premix (4µM) (Sigma, UK)	2.5
cDNA (1:10, cDNA : nuclease free H_2O)	2.5

Table 2.10 Volumes of components per reaction needed for quantitative real-time PCR (qPCR) to quantify target gene expression.

The amplification reactions were performed in triplicate in a clear 96-well plate (Bio Rad, UK). Serial dilutions of the control samples were also added to the 96 well plate in order to generate a standard curve. The plate was sealed with microseal plate sealers (Bio Rad, UK), vortexed briefly and centrifuged for approximately 1 min at 1800 rpm to collect and residual liquid and eliminate any air bubbles.

The plate was inserted into the CFX96 PCR Thermal Cycler (Bio Rad, UK) which was used to vary the temperature needed for qPCR to take place. Initially, the samples were heated to 98^{o}C for 2 min to activate the hot start DNA polymerase enzyme present in the SYBRgreen mix, they were then run for 40 cycles where the plate was heated to 98^{o}C for 2sec to denature the DNA and subsequently cooled to the optimum annealing temperature for 5sec which was required for the gene specific primer pair being used. The samples were further heated to 72°C for 10sec for elongation to take place and the cycle was repeated (figure 2.2).

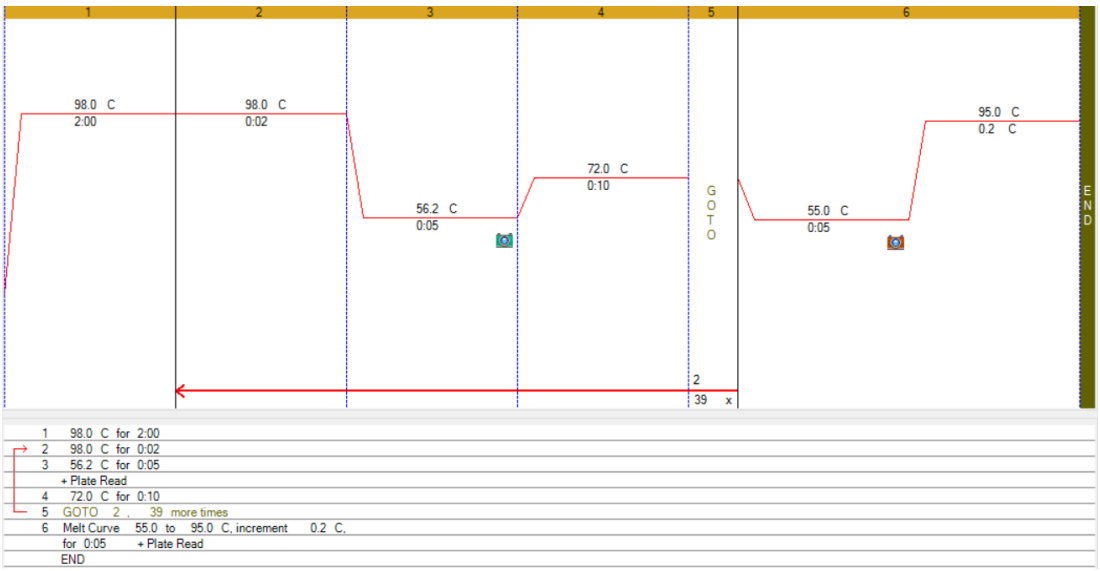


Figure 2.2 Typical Quantitative Real-Time PCR (qPCR) protocol layout used in the CFX96 PCR Thermal Cycler, where the current example is based on the optimum annealing temperature needed for Ribosomal Protein 60s L-19 (RPL-19) gene specific primers.

After the PCR reaction had taken place, a melt curve was generated, where the samples are held at least 1°C below the optimum annealing temperature of the gene specific primers being utilised for 5sec and then progressively the temperature was raised in increments of 0.2°C, until a temperature of 95°C was reached (figure 2.2). The data obtained from the qPCR and High Resolution Melt Analysis was saved to the Bio Rad CFX Manager Software 3.0 (Bio Rad, UK), and further analysis of the data was carried out using Microsoft Excel.

2.6.5 Detection of qPCR Products and qPCR output on CFX Manager 3.0

qPCR is a fluorescent-based method that allows for the detection and measurement of a specific starting quantity of complementary deoxyribonucleic acid (cDNA). The PCR reaction mix consists of SYBRgreen, gene specific forward and reverse primers and the sample of cDNA. SYBRgreen is a cyanine dye, which is able to bind and intercalate to double stranded DNA. The DNA-cyanine dye complex is excited at λ497 nm and emits green light at λ520 nm. This signal was detected in each PCR cycle and during the elongation step, the light emitted increases as the cyanine dye is able to bind more DNA, therefore the light emitted is proportional to the amount of DNA amplified by the gene specific primers. The intensity of the light emitted is measured in relative fluorescent units (RFU) and was plotted against the cycle number on a logarithmic scale. This is known as a

quantitative cycle plot, which takes on the appearance of a sigmoidal amplification curve. From this amplification graph, the quantification cycle (Cq) value for each sample was determined, which is the cycle number at which the relative fluorescence was considered higher than the background i.e. where the amplification curve intercepts the baseline threshold. This value was used to determine the starting quantity of DNA in the PCR reaction. The baseline threshold or where the signal was no longer background was automatically set by the Bio Rad CFX Manager Software, which was the level at where there was the earliest detectable signal but no product amplification (figure 2.3). This was usually a few cycles before the earliest signal crosses the threshold. The fluorescence, which was detected below the baseline threshold, represents noise, for example non-specific signal from inefficient binding of SYBRgreen.

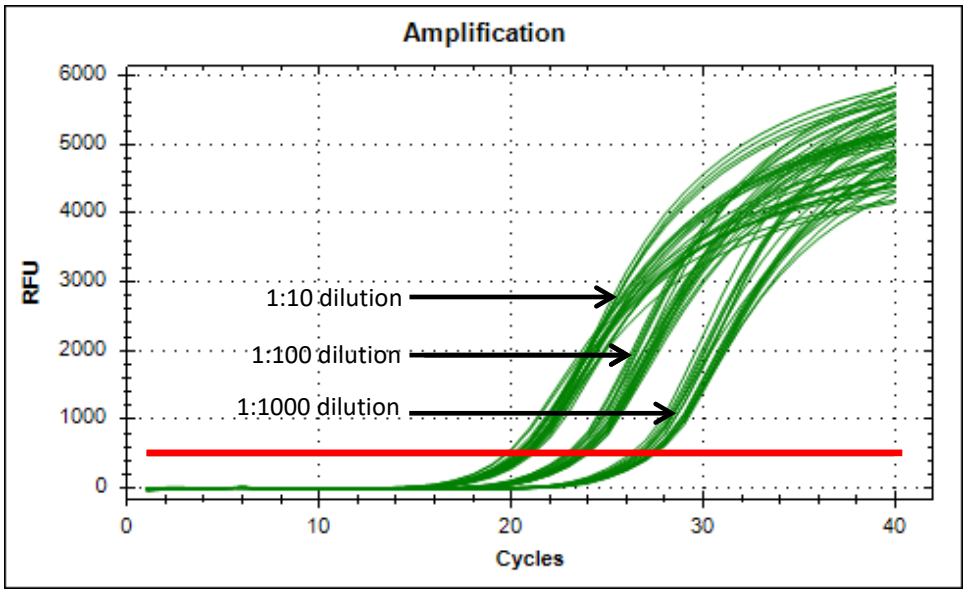


Figure 2.3 Amplification plot from Quantitative Real-Time PCR (qPCR) This example shows an amplification plot generated with specific primers for the internal reference gene; RPL-19 in ESCs isolated from fertile patients. The serial dilutions, which are used to generate the standard curve, are indicated. The red line specifies at which point the fluorescence signal was considered higher than the background.

The likelihood that it was due to primer dimers, contamination or incorrect annealing of the primers, was eliminated by the fact that a melt curve was produced by the software. To obtain a melt curve for each sample, the samples are held at least 1°C below the optimum annealing temperature for the gene specific primers being utilised for 5 sec and then progressively the temperature was raised in increments of 0.2°C until a temperature of 95°C

was reached. This slowly denatures the bonds between the bases of the product, which will alter its fluorescent properties. When the change in RFU was plotted against the temperature (^{o}C), a peak of fluorescence was detected at a specific temperature, which was used to check the specificity of the PCR reaction (figure 2.4).

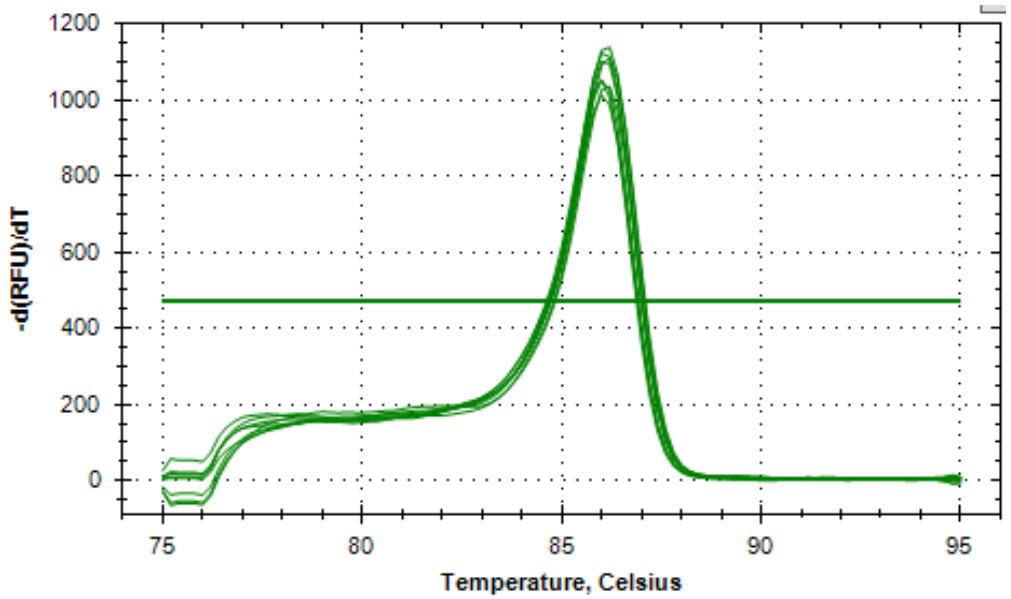


Figure 2.4 Melt curve output from a typical quantitative real-time PCR reaction. This example shows a melt curve generated with Ribosomal Protein 60s L-19 (RPL-19) gene specific primers against cDNA from ESCs isolated from fertile patients.

2.6.6 Generation of a Standard Curve for qPCR Data Analysis

In order to effectively analyse qPCR data and to check the efficiency of the primers, standard curves were generated. Serial dilutions of the control sample were made as previously described (1:5, 1:10, 1:100 and 1:1000 – cDNA: H2O) for each gene evaluated in this project. 2.5 µL of these dilutions was added to 2.5 µL of the gene specific forward and reverse primer premix and 5 µL of SYBRgreen. The samples in triplicate were run on the CFX96 PCR Thermal Cycler and the Cq values are generated for each sample. These Cq values are plotted against the logarithm of the starting quantity of each standard to generate a standard curve. These standard curves were used to relatively quantify gene expression from the Cq values obtained. An example of a standard curve generated using the CFX Manager 3.0 for the internal reference gene; RPL-19 in ESCs isolated from fertile patients is seen in figure 2.5. A new standard curve was produced on each qPCR run.

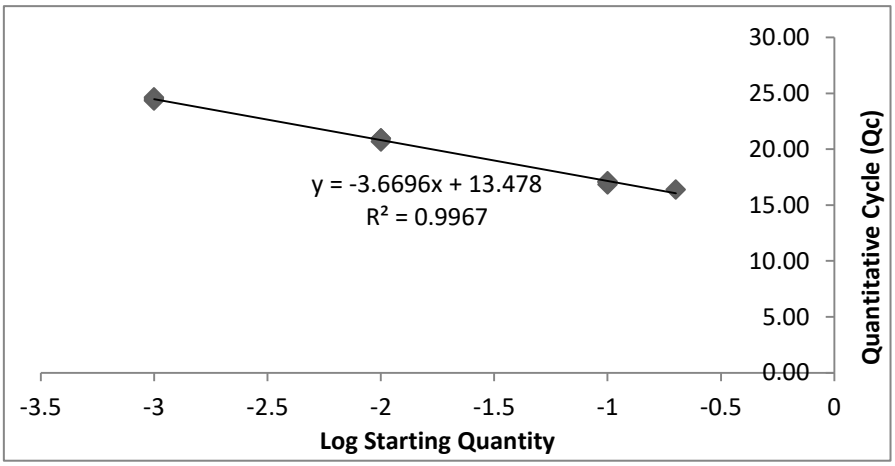


Figure 2.5 Typical standard curve obtained from the CFX Manager 3.0 output for the internal reference gene; RPL-19, from ESCs isolated from fertile patients.

The standard curves produced for each gene under investigation were checked for validity, where the gradient of the standard curve had to lie between -3.3 and -3.8. If the standard curve did vary greatly from these values, they were discarded and new standard curves were generated for the gene of interest. The gradient of the standard curve is a good indicator of the efficiency of the qPCR reaction, as if the qPCR reaction was 100 % efficient (or R^2=1.00), the gradient of the standard curve would equal -3.322, which indicates that the qPCR product was being doubled in each cycle (Thermofisher, UK). However, due to experimental limitations, this value has a low probability of being achieved; therefore, a slope with an R^2 value between 0.95 and 1.00 is satisfactory, which indicates that there is a strong correlation between the logarithm of the starting quantity and the Cq number. These values were achieved in the typical standard curve obtained for all genes tested, including the internal reference gene; RPL-19 in ESCs isolated from fertile patients (figure 2.5).

2.6.7 Quantitative Real-Time PCR Data Analysis using Microsoft Excel

Relative quantification of gene expression was determined from the triplicate Cq values for each sample, which were copied and pasted from the CFX Manager 3.0 into Microsoft Excel as raw data. The average of the triplicate values was calculated and if necessary, one outlying Cq value per triplicate was excluded from the analysis and the average re-calculated. Subsequently, the standard curve was generated and the data acquired from the linear equation of the curve was used to calculate the log of the starting quantity. The log of the starting quantity was calculated using the following equation:

$$Y = \frac{X - Z}{M}$$

Where Y is the logarithm of the starting quantity, X is the average of the triplicate Cq values, Z is the gradient of the standard curve and M is the curve intercept on the Y axis. The starting quantity for each sample was calculated by using the Power (10, Y), where Y is the logarithm of the starting quantity. The starting quantity of the target gene in each sample was consequently normalised against the starting quantity obtained for the endogenous reference target gene, RPL-19. This was achieved by dividing the starting quantity of the gene of interest by the starting quantity of the reference gene. Gene expression was further calculated as a ratio of transcript levels between the untreated (control) and treated samples to gain the fold expression change.

All qPCR experiments were performed in triplicate and all were subject to statistical analysis. The normalised starting quantity of treated samples were statistically compared to the untreated control samples and also to samples treated with various other treatments using the two-tailed student's T-test if data was normally distributed or if the data was not normally distributed, the Kruskal-Wallis or Mann-Whitney U test was used. After analysis, results were considered statistically significant when $p \leq 0.05$, 0.01 or 0.001 – which is denoted by the presence of *, ** or *** respectively on the graph. All statistical data analysis was performed using SPSS (SPSS, Chicago, Illinois).

2.7 RNA and Protein Isolation from Fresh Biopsies

The TissueRuptor (Cat: 9001273, Qiagen, UK) which is a rotor-stator homogenizer was used to thoroughly disrupt and homogenise tissue, in the presence of the appropriate lysis buffer (either RIPA buffer or RLT lysis buffer in order to extract protein and RNA respectively). The sample was homogenised using the TissueRuptor disposable probes (Cat: 990890, Qiagen, UK) following the recommended manufacturer extraction protocol. The rotor-stator rotates at very high speeds, which causes the tissue sample to be disrupted and homogenised by both mechanical shearing and turbulence. Disruption of the sample was necessary to release either the nucleic acids or protein contained in the sample, where incomplete disruption results in reduced yields. Homogenization was necessary to reduce the viscosity of the cell lysates produced by disruption, where homogenization shears the high molecular-weight cellular proteins and carbohydrates to create a homogenous lysate. Incomplete homogenization results in significantly reduced yields for either protein or RNA extraction. Due to the high speeds, foaming of the sample can occur, however this should be kept to a

minimum by keeping the tip of the disposable probe submerged at all times, using the appropriate sized vessel and the tip of the probe should be held towards the side of a round bottom tube rather than a conical bottom tube. If foaming did occur, the sample was left on ice for approximately 5 min to allow the foam to settle. A disposable probe with a diameter of 5–7 mm was used which are suitable for volumes of up to 300 µL. For RNA extraction, RLT lysis buffer was used to disrupt and homogenise the sample, followed by RNA extraction using the RNeasy kit (Qiagen, UK) and on column DNAse I digestion, and finally RNA was eluted using nuclease free water (seen in section 2.6.1). The volume of RLT buffer used depends upon the size of the tissue sample, which cannot be greater than half the diameter of the TissueRuptor disposable probe. If the sample was larger than this, the sample must be cut into smaller pieces before being disrupted. A minimum of 180 µL of RLT buffer was used and the sample was disrupted for approximately 20 sec at full speed. For optimal results, the sample should be homogenised for the minimum amount of time possible and the tip of the probe should be moved within the sample during homogenisation to increase the efficiency of disruption. After successful homogenisation, the lysate was transferred to a Qiashredder spin column and the protocol described in section 2.6.1 was followed. Alternatively, for the extraction of protein from the biopsy tissue, approximately 30 mg of tissue sample was used and if the sample was greater in size than half the diameter of the TissueRuptor probe, the sample should be cut into smaller pieces. A minimum of 180 µL of RIPA buffer containing phosphatase and protease inhibitors should be used and the TissueRuptor probe tip should always be submerged in the buffer. The sample was disrupted for approximately 30sec at full speed with the probe being moved within the sample during homogenisation to increase the efficiency of disruption. The lysate was then processed according to the protocol described in section 2.5.

2.8 Enzyme-Linked Immunosorbant Assay (ELISA)

2.8.1 Quantification of Secreted Decidual Prolactin (dPRL) and Insulin-like growth factor-binding protein 1 (IGFBP-1) in Cell Culture Media

Cell media from confluent monolayers of HESC and ESC cells subjected to decidualization and GR activation treatments was collected and stored in aliquots of 500 µL at -20^{o}C or -80^{o}C for long-term storage. The cell monolayers were used to extract RNA or protein as described in section 2.5 and section 2.6.1 respectively. This cell supernatant was subsequently used in a commercially available enzyme-linked immunosorbant assay (ELISA). The concentration of secreted prolactin (PRL) (Cat No: DY682, R&D Systems,

USA) and Insulin-like growth factor-binding protein 1 (IGFBP-1) (Cat No: DY871, R&D Systems, USA) using commercially available ELISA kits and the manufacturers protocol was followed for both. All the antibodies described in the following protocols were provided with the kit.

PRL or IGFBP-1 monoclonal capture antibody was added to a 96 well ELISA plate (Cat No: 650001, Greiner Bio-One, UK) after diluting the antibodies to their working concentration with PBS. The plates were immediately coated with the antibody solution and sealed with a polyester film (Cat No: 391-1250, VWR, UK). The plate was incubated overnight on a rocker at room temperature, which allowed the capture antibody to successfully bind to the plate. Each well was aspirated and washed with wash buffer, which consists of 0.05 % Tween®20 (Sigma, UK) in PBS (Gibco, UK) and this process was repeated three times, where complete removal of liquid was essential for good performance of the ELISA kit. After the last wash step, any remaining wash buffer was removed by inverting the plate and blotting against clean paper towels. The ELISA plate was subsequently blocked using blocking buffer (or reagent diluent), which consisted of 1 % BSA in PBS for the PRL ELISA and 5 % Tween®20 in PBS for the IGFBP-1 ELISA. The plate was blocked for a minimum of 1 h at room temperature, to ensure that all unbound sites on the plate were blocked and to prevent any false positive results. The washing step was repeated and the wells were aspirated well and the plate was then ready to perform the assay.

To quantify the concentration of either secreted PRL or IGFBP-1 in cell culture media, known concentrations of these two proteins were used to generate a 7-point standard curve. The standards were provided with the ELISA kits, where they contained 150 ng/mL of recombinant human PRL standard and 118 ng/mL of recombinant human IGFBP-1 standard. The PRL and IGFBP-1 standards were serially diluted using the previously described block buffer (or reagent diluent) for each assay, to concentrations 1000, 500, 250, 125, 62.5, 31.25 and 15.625 pg/mL and 2000, 1000, 500, 250, 125, 62.5 and 31.25 pg/mL respectively to be used in the assays. Block buffer (or reagent diluent) which was used for the serial dilutions was prepared without standard and used to account for background and was consequently subtracted from each reading. Along with the standards, the samples were also added to the 96 well plates and incubated for 2 h at room temperature. This allowed for either the prolactin or IGFBP-1 protein to bind to the capture antibody which has been previously bound to the plate. After this incubation step, any excess of the samples or standards was decanted and the previously described wash steps were repeated to remove any unbound protein.

Following this, the detection antibody was added to each well, which is a biotinylated secondary antibody which contained goat anti-human prolactin or goat anti-human IGFBP-1. The plate was incubated with the secondary antibody for 2 h at room temperature to enable the antibody to bind to the target protein, whether it is prolactin or IGFBP-1. The previously described wash step was then repeated and the wells were aspirated well. Streptavidin-horseradish peroxidase (HRP) was consequently added to the plate and incubated for 20 min at room temperature, ensuring the plate was not in direct sunlight. The previously described wash step was then repeated and the wells were aspirated.

A substrate solution was added to the plate, which contains hydrogen peroxide and tetramethylbenzidine, which is able to react with the HRP present on the plate and results in a blue colour developing which is directly proportional to the concentrations of target protein present in the standard or samples. To stop the reaction, Sulphuric acid (H_2SO_4) (1 M) was added to the plate resulting in a colour change from blue to yellow. Finally, the optical density of each well was determined using the FLUOR star OPTIMA plate reader (Labtech, UK) at an absorbance of λ450 nm. The standards of known concentration for both prolactin and IGFBP-1 were used to prepare the 7-point standard curve and concentration of protein present in the samples was calculated using the equation; $y = mx + c$, which is the equation of the straight line from the known standard concentrations and inputting the values obtained for the unknown samples into this equation.

2.8.2 Quantification of Circulating Levels of Hormones in Patient Serum samples

All circulating levels of hormones were measured in serum samples isolated from patients recruited to this study. Whole blood was collected from consenting patients in ABMUHB hospital and transported to Swansea University in serum (clot activator) tubes (BD Company, UK). These tubes contain both silica particles, which activate clotting and a gel to separate the serum. After collection of the whole blood, the blood was allowed to clot at room temperature. The clot was then removed by centrifugation at 1000 xg for 10 min. The resulting supernatant is the serum, and this was consequently aliquoted and stored at -20^{o}C and -80^{o}C. Freeze-thaw cycles were avoided, as these are detrimental to serum components.

The isolated serum was used to determine the concentration of several circulating hormones using commercially available ELISA kits and the manufacturer's protocols were followed. All the antibodies described in the following protocols, were provided with the kits.

2.8.2.1 Cortisol (Compound F) and Cortisone (Compound E)

The concentration of circulating cortisol (compound F) and cortisone (compound E) was determined in the serum using a commercially available ELISA kit (Cat No: KGE008B, R&D Systems, USA and Cat No: K017-H1, Arbor Assays) and the manufacturers protocol was followed.

A pre-coated goat anti-mouse polyclonal antibody 96 well plate was used for the cortisol assay and a pre-coated goat anti-rabbit IgG antibody 96 well plate was used for cortisone assay. Calibrator diluent RD5-43 or assay buffer was added into the non-specific binding wells and the zero standard wells for the cortisol and cortisone assay respectively. Cortisol and cortisone standards were prepared and serum samples were pre-treated to remove potentially interfering proteins and protein-bound cortisol and cortisone. The standards and pre-treated serum samples were added to the plate and consequently cortisol or cortisone conjugate was added to all wells which resulted in the wells becoming red in colour. Mouse monoclonal primary antibody was added directly after the cortisol conjugate to all wells (except the non-specific binding wells), which resulted in a colour change from red to violet (except the non-specific binding wells which remained red in colour) and the plate was sealed with a polyester film (Cat No: 391-1250, VWR, UK). In the cortisone assay, a rabbit polyclonal antibody was added directly after the cortisone conjugate to all wells. The plates were incubated for 2 h at room temperature on a rocker to ensure sufficient binding of the primary antibody to cortisol or cortisone. Each well was aspirated and washed with wash buffer, which consists of a buffered surfactant in deionised water and this process was repeated four times, where complete removal of liquid was essential for good performance of the ELISA kit. After the last wash step, the plate was aspirated well and any remaining wash buffer was removed by inverting the plate and blotting against clean paper towels. Substrate solution was prepared for the cortisol assay by adding equal volumes of colour reagent A and colour reagent B and added to each well which contains hydrogen peroxide and tetramethylbenzidine. Whereas in the cortisone assay, pre-prepared TMB substrate was added to each well. The plates were incubated for 30 min at room temperature, ensuring the plate was not in direct sunlight. To stop the reaction, after 30 min, stop solution (1 M sulphuric acid for cortisol and 1M hydrochloric acid for cortisone) was added to each well, which results in a colour change from blue to yellow.

Finally, the optical density of each well was determined using the FLUOR star OPTIMA plate reader (Labtech, UK) at an absorbance of λ450 nm and wavelength correction was set at λ540 nm. These values were subtracted from the readings at λ450 nm, which correct for optical imperfections in the plate. The duplicate readings were averaged for each standard

and sample and the average non-specific binding optical density values were subtracted from these. The standards of known concentration for cortisol and cortisone were used to prepare the 7-point standard curve and concentration of protein present in the samples was then calculated using the equation; y = mx + c, which is the equation of the straight line from the known standard concentrations and inputting the values obtained for the unknown into this equation. Since samples had been diluted, the concentration read from the standard curve was multiplied by the dilution factor (x60 for cortisol and x100 for cortisone).

2.8.2.2 Progesterone (P_4)

The concentration of circulating progesterone (P_4) was determined in the serum using a commercially available ELISA kit (Cat No: 582601, Cayman Chemical) and the manufacturers protocol was followed.

A pre-coated mouse anti-rabbit IgG antibody 96 well plate was used. ELISA buffer was added to the non-specific binding and zero standard wells. Progesterone standards were prepared and the standards and serum samples were added to the plate. Consequently, a progesterone acetylcholinesterase (AChE) tracer was added and directly afterwards, progesterone ELISA antiserum was added to all wells (except the non-specific binding and blank wells), as this assay is based on the competition between progesterone present in the samples with the progesterone tracer for a limited number of progesterone-specific rabbit antiserum binding sites. The plate was sealed with a polyester film and incubated for 1 h at room temperature on a rocker. After 1 h , Ellman's reagent was reconstituted and prepared. Each well was aspirated and washed with wash buffer and this process was repeated four times, where complete removal of liquid was essential for good performance of the ELISA kit. After the last wash step, the plate was aspirated well and any remaining wash buffer was removed by inverting the plate and blotting against clean paper towels. Ellman's reagent was added to each well. The plate was incubated for 90 min at room temperature on a rocker and protected from sunlight. Finally, the optical density of each well was determined using the FLUOR star OPTIMA plate reader (Labtech, UK) at an absorbance of λ420 nm.

The duplicate readings were averaged for each standard and sample and the average non-specific binding optical density values were subtracted from these. The standards of known concentration for progesterone were used to prepare the 7-point standard curve and concentration of protein present in the samples was then calculated using the equation; y = mx + c, which is the equation of the straight line from the known standard concentrations and inputting the values obtained for the unknown into this equation.

2.8.2.3 Estradiol (E_2)

The concentration of circulating estradiol (E_2) was determined in the serum using a commercially available ELISA kit (Cat No: KGE014, R&D Systems, USA) and the manufacturers protocol was followed.

A pre-coated goat anti-mouse IgG antibody 96 well plate was used. Estradiol standards were prepared and serum samples were pre-treated to remove potentially interfering proteins and protein-bound estradiol. Estradiol primary antibody was added to each well (except the non-specific binding wells) and the plate was sealed with a polyester film and incubated for 1 h at room temperature on a rocker to ensure sufficient binding of the primary antibody to the plate. Each well was aspirated and washed with wash buffer, which consists of a buffered surfactant in deionised water and this process was repeated four times, where complete removal of liquid was essential for good performance of the ELISA kit. After the last wash step, the plate was aspirated well and any remaining wash buffer was removed by inverting the plate and blotting against clean paper towels. The standards and pre-treated serum samples were added to the plate and calibrator diluent RD5-62 was added into the non-specific binding wells and the zero standard wells. Estradiol conjugate was added to all wells and the plate was sealed with a polyester film and incubated for 2 h at room temperature on a rocker. The previous wash step was repeated and after the last wash step, the plate was aspirated well and any remaining wash buffer was removed by inverting the plate and blotting against clean paper towels. Substrate solution was prepared by adding equal volumes of colour reagent A and colour reagent B and added to each well which contains hydrogen peroxide and tetramethylbenzidine. The plate was incubated for 30 min at room temperature, ensuring the plate was not in direct sunlight. To stop the reaction, stop solution (1M Sulphuric acid) was added to each well and the optical density of each well was determined and the concentration of estradiol present in the unknown samples was determined as described in section 2.8.2.1.

2.8.2.4 Androgens, LH and FSH levels

The serum levels of testosterone, the sex hormone binding globulin (SHBG), dehydroepiandrosterone sulfate (DHEA sulfate), LH and FSH levels were measured by electro-chemiluminescence immunoassay (ECLIA), using an Elecsys 2010 immunoassay analyser (Roche) at the ABMUHB biochemistry department.

Testosterone was determined using the Elecsys® Testosterone II assay. The lower limit of the assay quantitation was 0.416 nmol/L (0.120 ng/mL) and the intra-assay and inter-assay coefficient of variation (CV) was 1.2- 4.7 % and 2.8-8.4 % respectively. SHBG was measured by the Elecsys® SHBG assay (Roche). The lower limit of quantitation of the assay was 0.350 nmol/L (33 ng/mL) and the intra-assay and inter-assay CV was 2.1-2.7 % and 2.6-5.6 % respectively. DHEAS was measured by the DHEAS Elecsys® assay (Roche). The lower limit of quantitation was 0.003 μmol/L (0.10 μg/dL), and the intra-assay and inter-assay CV was 1.7-2.8 % and 2.4 – 4.7 % respectively.

LH was measured by Elecsys® LH assay (Roche). The lower limit of quantitation of the assay was 0.100mIU/mL, and the intra-assay and inter-assay CV was 0.8-1.8 % and 1.9-5.2 % respectively. FSH was measured by the Elecsys® FSH assay (Roche). The lower limit of quantitation was 0.100mIU/mL, and the intra-assay and inter-assay CV was 1.4-2.0 % and 2.9-5.3 % respectively.

Androstenedione was assayed using a radioimmunoassay (RIA) (Beckman Coulter), the lower limit of quantitation of the assay was 0.698 nmol/L (0.2 ng/mL) and the intra-assay and inter-assay CV was 5.7 % and 10.6 % respectively.

2.9 Cell Imaging using Zeiss Light Microscope and Image J Analysis

The Zeiss light microscope (Zeiss, Germany) was used for monitoring cell morphology and to check their levels of confluence. The Zeiss imaging system (Zen Blue Edition, Carl Zeiss Microscopy) was used to take still images of the cell monolayers after 48h of treatments. The images of the treated cell monolayers in the 6 well plates were taken at x10 and x20 magnification.

Images were then subjected to Image J analysis to analyse cell morphology, where the circularity of the ESCs (n=30 per patient) was calculated using Image J software. Cell circularity is based on the equation (Image J Circularity, 2016).

$$\text{Circularity} = 4\pi \text{ (area in pixels/perimeter}^2\text{)}$$

This equation generates a value from 0-1, where 1 indicates a perfect circle and as the value approaches 0, this indicates an increasingly elongated polygon (Image J Circularity, 2016).

2.10 Promoter Analysis

Promoter analysis was carried out using TRANSFAC online database (GeneXplain GmbH, Germany). TRANSFAC is a transcription factor (TF) database which includes information on over 8000 TFs and over 18,000 TF binding sequences found in vertebrates, bacteria, fungi, insect, nematode and plant genomic sequences. TRANSFAC online database requires subscription for access and is distinct from the publicly available TRANSFAC databases as it includes the most up to date releases of TFs and their binding sites and allows for specialised searches to be made. The work presented here was performed using the TRANSFAC online subscription only database.

TRANSFAC uses both experimental and bioinformatics evidence of TF binding sites to construct nucleotide positional weight matrices which measure the relative likelihood that a TF binds to a particular input sequence. The matrices produced in these databases are constructed by aligning multiple known TF binding sequences and recording the frequency that each nucleotide occurs at each position. The five most highly conserved consecutive bases are designated as the 'core' binding sequence. Many matrices are constructed using the evidence of experimental studies in a particular species, and also by collating evidence of publications often across several species. Therefore a TF will have multiple matrices associated with it, which relates evidence from different studies and species.

In this study, TRANSFAC was used to interrogate the promoter region of GR and its potential target genes to identify potential TF binding sites activated by in vitro decidualization and the GR signalling pathway.

2.10.1 Analysing Promoter Sequences using TRANSFAC Matrices

TRANSFAC online includes a module called 'Match' analysis, which accepts input nucleotide sequences to be searched for the alignment with TF matrices. Promoter sequences (3kb long) corresponding to GR and the potential GR target genes were extracted either using the TRANSFAC database if the promoter sequence (using best supported promoter only) was available or using the UCSC Genome Browser Gateway using the hg38 assembly (University of California, Santa Cruz) and consequently analysed using the Match module.

When using the Match module, the following search parameters were applied. Only groups of matrices relating to vertebrate TFs were considered in the search. Additionally, only 'high quality' matrices were considered, these are defined as matrices which generate less than 10 hits per 1000 nucleotides in test alignments with promoter sequences by GeneXplain, this therefore discredits approximately 5% of the matrices within the TRANSFAC database which would have generated a high number of false positive results. The similarity score parameter was also adjusted whilst performing the promoter analysis. TRANSFAC searches for motifs within the query sequences which pass a threshold of similarity with the 'core' and 'matrix' consensus sequences. The maximum core or matrix score available is 1.0 and for the purpose of this study the similarity thresholds were set at the recommended optimal values of 0.75 for both core and matrix sequences.

2.10.2 Transcription Factor (TF) Binding Site Analysis

The results generated using the high quality matrices in the Match analysis were consequently interrogated to find candidate TF binding sites for GR on the promoter of NR3C1 and GR potential target genes. Additionally, potential TF binding sites for the proposed GR targets on the promoter of NR3C1 were analysed.

The consensus binding sites for GR according to the literature are glucocorticoid response elements (GREs), negative GREs (nGREs), SP-1 and AP-1.

This work focused on GR binding to the GRE sequences within the promoters of target genes, where this work was used in downstream analysis using chromatin immunoprecipitation to identify protein-DNA interactions.

2.11 Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was conducted using the Chromatrap® ChIP-seq solid phase isolation technique (Cat No: 500189 and 500190, Chromatrap, UK), according to the manufacturer's guidelines. The ChIP process involves the fixation or cross-linking and consequent isolation of the chromatin (DNA-protein complexes) from the nucleus of the cells. Consequently, the chromatin was broken into smaller fragments between 100 – 500bp by sonication. Transcription factor (TF) complexes of interest were selected by IP using specific antibodies for the target TF complex. The cross-linking was then reversed and the protein transcription complex was digested, the genomic DNA was detected using qPCR,

ensuring that a negative control was included, to ensure that the amplification of the target DNA was selective and specific.

2.11.1 Chromatin Isolation

After 48h of treatment following the protocol described in section 2.4.2., the cell culture media was decanted and the cell monolayer was washed twice with 8mL of PBS and removed. 8mL of fixation medium which contains 1% formaldehyde (Cat No: 252549, Sigma, UK) in basic media (containing no serum or large molecular weight proteins) was added to the flask. The cell monolayers (1000-15million cells) were incubated with the fixative for 10min at room temperature on a rocker. This enables cross links between the DNA/protein and protein/protein to occur. The fixation media was subsequently removed and replaced with 8mL of glycine solution (0.65M) and incubated for 5min at room temperature on a rocker, which quenches the reaction. The cells were then harvested by decanting the glycine solution and 8 mL of ice cold PBS was added to each flask. The cell monolayer was consequently scraped and the lysate was added to a clean 15mL centrifuge tube. The samples were centrifuged at 3500xg for 5min at 4^{o}C to pellet the cells. The supernatant was decanted and the pellet was re-suspended and 400µL of hypotonic buffer, gently mixed by pipetting and incubated on ice for 10min to lyse the cells. The hypotonic slurry was centrifuged at 5000 xg for 5min at 4^{o}C to collect the intact nuclei fraction. Following the removal of the supernatant, the nuclei pellet was re-suspended in 300 µL of lysis buffer, gently mixed by pipetting and incubated again on ice for 10 min. To obtain chromatin which contains DNA fragments of 100 – 500 bps, the samples were subjected to sonication for 15 min using pulses of 30sec at an amplitude of 50, followed by a rest period of 30sec. The chromatin samples were centrifuged at 20000 xg at 4^{o}C to remove any nuclear debris material and the supernatant was transferred to a clean Eppendorf tube and 1µL of the protease inhibitor cocktail was added and mixed. All chromatin samples were stored at – 80^{o}C.

In order to check the efficiency of sonication and to quantify the amount of chromatin harvested from each sample, 25µL aliquots of stock solutions were taken and processed for agarose gel electrophoresis and nanodrop analysis respectively. In order to process the samples, they were firstly reversed cross-linked and purified. To reverse cross-link, the samples (25µL) are incubated overnight with 5µL of $NaHCO_3$ (1M), 5 µL of NaCl (5M) and 15µL of dH_2O at 65 ^{o}C. After the overnight incubation, the samples are incubated for 1h with 1µL of proteinase K at 37^{o}C to degrade any proteins remaining. The samples were consequently returned to room temperature and 2µL of Proteinase K stop solution was

added. The DNA was quantified using a spectrophotometer set to 260nm (Nanodrop 2000c Thermo Scientific). The reading was multiplied by a factor of 2 to account for the dilution during the reverse cross-linking, which will be used to determine the volume of chromatin to load on IP columns. To ensure that 100-500bp fragments had been obtained during shearing, the DNA was run on a 1% agarose gel and visualised against the marker with DNA fragments of known size (Promega, UK). A smear of DNA fragments of 100-500 bp in length was optimal, fragments which are either smaller or greater in length may affect the efficiency of the ChIP reaction (figure 2.6).

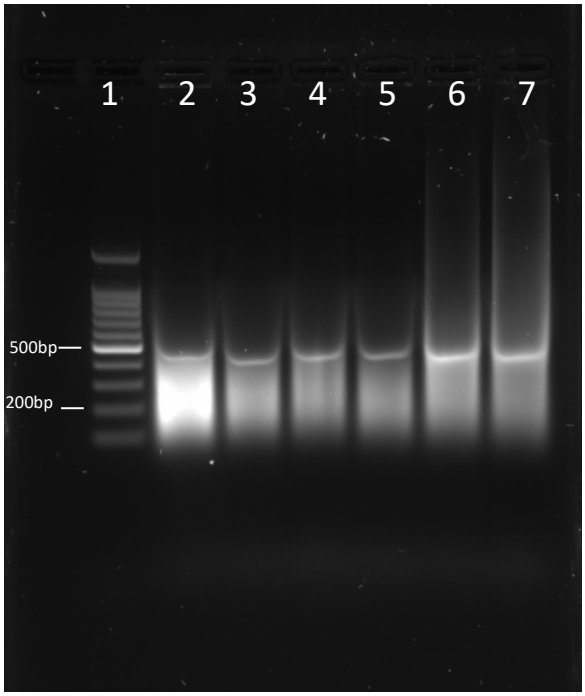


Figure 2.6 Gel Analysis of optimal shearing by sonication of chromatin samples. The human endometrial stromal cell line (HESC) samples were fixed with 1% formaldehyde. Chromatin was sheared using a sonicator. Chromatin was subjected to reverse cross-linking and run on a 1% agarose gel and visualised. Lanes 2 – 7 contain the HESC samples and the lane 1 contains the DNA ladder with fragments of known size, indicating where the 500bp and 200bp marker are located.

2.11.2 Immunoprecipitation (IP) After Chromatin Isolation

Following chromatin isolation and sonication, the stocks were added to an immunoprecipitation (IP) slurry mixture for each reaction. The IP slurries were made for each samples with the antibody of interest; GR (H-300) (Cat No: sc-2880, Santa Cruz, USA), along with an isotype control IgG; Anti-Rabbit IgG (Cat No: ab171870, Abcam, UK) and a DNA input, which was not processed through the IP columns. ChIP reactions contained 1 µL of protease inhibitor cocktail (PIC), 5µg of sheared chromatin, 25µL of dH_2O and 2.5µg of the antibody of interest and column conditioning buffer was used to

make the final volume of the slurry to 1mL. The IP slurries were incubated for 1 h at 4°C on an end to end rotor. During this time, the Chromatrap® spin columns were prepared by washing through 600 µL of column conditioning buffer twice. After 1h the slurries were removed from the end to end rotor and spun down to remove residual liquid from the lids. The entire 1 mL slurry was added to the columns and let to flow through. The spin columns were put in collection tubes and were washed twice with 600µL of wash buffer 1, was buffer 2 and wash buffer 3 and centrifuged at 4000xg for 30sec, the flow through was discarded between each wash step being repeated. After the wash steps, the columns were spun dry using a centrifuge at top speed for 30sec to remove and remaining liquid from the columns. The columns were then added to a clean 1.5mL Eppendorf tube and 50µL of ChIP-seq elution buffer was added and incubated for 15min at room temperature. After the incubation, the columns were centrifuged at top speed for 30sec to collect the eluted chromatin. It is important to note that the input was placed on ice during this time.

Chromatin samples, then needed to undergo reverse cross-linking to release the DNA from the protein bound complexes. This was done by adding 5µL of $NaHCO_3$ (1 M), 5µL of NaCl (5 M) and 50µL of dH_2O was added to each eluted chromatin sample and input and incubated overnight at 65°C, to facilitate reverse cross-linking. After this incubation step, 1µL of proteinase K was added for 1h at 37°C to degrade any bound antibody – protein complexes in the sample. After 1h, 2µL of proteinase K stop solution was added to eluted chromatin samples and inputs.

Chromatin was then purified using the Chromatrap® DNA purification columns provided with the ChIP-seq kit. Briefly, 5 volumes of DNA binding buffer was added to 1 volume of the sample and mixed. The DNA binding buffer contains a pH indicator, therefore if the pH > 7.5, the mixture turns orange or violet, which means that the pH of the sample exceeds the buffering capacity of the DNA binding buffer and DNA adsorption will be inefficient. In the cases that this happened, 10µL of sodium acetate (3M) at pH 5 was added to adjust the pH of the binding mixture and the mixture changes colour to yellow. This sample was added to a DNA purification column and centrifuged at 16,000xg for 60sec and the flow through was discarded. After centrifugation, 700µL of DNA wash buffer was added to the column and centrifuged at 16,000 x g for 60 sec and the flow through was discarded. The column was centrifuged at 16,000 x g for 60 sec for a dry spin, to remove residual wash buffer. The DNA purification tube was added to a new 1.5mL Eppendorf tube and 50µL of DNA elution buffer was added to the column membrane and incubated for 1min. After the incubation, the columns were centrifuged at 16,000xg for 60sec. The eluted DNA samples are now ready for validation by qPCR. qPCR reactions were performed as described in section 2.6.4. Specific genomic primers were designed using Beacon Design 8.0 programme (Premier Biosoft,

USA) to amplify specific genomic binding regions (table 2.11). The analysis of the output data is described below

Gene – Promoter Binding site	Primer	Primer Sequence (5'-3')	(Ta) / ^{o}C
NR3C1 – GRE binding site (-3214/-3196)	Sense	TTCCGTAAGCACATATTG	60
	Anti-sense	TGAGATAGGTATTGTTATTCG	
PRL – GRE binding site (-1413/-1395)	Sense	TTTCCCAGCCTTCCTCAT	55
	Anti-sense	CGCACCAGCCTAATAATTCTAT	
WT1 – GRE binding site (-846/-828)	Sense	TCAAACTTCAGGAGGGACTTA	55
	Anti-sense	GAGCACAATCACTCTGTAACA	
FKBP5 – GRE binding site (-2328/-2310)	Sense	GTCCCATCACAGAGAGTA	55
	Anti-sense	TGTATGTTATCAACTTCAGACT	
PGR – GRE binding site (-1898/-1880)	Sense	CACCTTCTGGAGTCTTCT	54
	Anti-sense	AAACAGAGTGAATGATTTGC	
AR – GRE binding site (-3013/-2995)	Sense	TGTTGACAGCACCATCTT	54
	Anti-Sense	CCTCAGAAGGCTAGTGTG	
HSD11B1 – GRE binding site (-2700/-2682)	Sense	GGGTGGTTGAGCAGAAAT	54
	Anti-Sense	TGGATGGACTAACAATTAACTGA	
HSD11B2 – GRE binding site (-2667/-2649)	Sense	CAACAGGAATCAGTGACT	56.3
	Anti-Sense	GTGTTTCTGGTAACTCTCT	

Table 2.11 Genomic primer sequences for GRE sites in the NR3C1, PRL, WT1, FKBP5 PGR, AR, HSD11B1and HSD11B2 promoter sequences retrieved from TRANSFAC analysis.

2.11.3 Analysis of qPCR data post ChIP

After the qPCR reaction was completed, the average Cq value for the antibody, isotype control IgG and the input were calculated from the triplicate wells. An antibody percentage signal relevant to the input amplification and PCR efficiency was calculated along with the equivalent isotype control IgG percentage value. The real signal can be calculated by deducing the IgG percentage from the target antibody percentage. Statistical analysis was carried out using data from a minimum of three independent repeats.

2.12 Statistical Analysis

The normality of the data was assessed using the Kolmogorov-Smirnov test. Normally distributed data was analysed using an ANOVA test followed by the parametric student's t-test to determine statistically significant differences between data groups. Whereas, non-normally distributed was analysed using the non-parametric Kruskal Wallis test followed by a Mann-Whitney test to determine significant differences between specific groups. Results were considered statistically significant, highly statistically significant and very highly statistically significant when $p \leq 0.05$, 0.01 or 0.001 – which is denoted by the presence of *, ** or *** respectively. All statistical data analysis was performed using SPSS (SPSS, Chicago, Illinois).

CHAPTER 3

The expression of the Glucocorticoid Receptor (GR) in the human endometrium of fertile and infertile patients

3.1. Introduction

The endometrium is a complex tissue comprising of mesenchymal-derived stroma, glandular and luminal epithelia, vascular smooth muscle and leukocytes. It's unique cyclical disintegration, regeneration and differentiation is closely regulated by the pituitary released gonadotropins and the ovarian derived sex steroid hormones (Chan, 2004). During the proliferative phase of the menstrual cycle, the endometrium responds to estrogen which is released during ovum maturation prior to ovulation. Estrogen promotes the regeneration and proliferation of the endometrium through the estrogen receptor (ER) and also up-regulates progesterone receptor (PR) expression, allowing the endometrium to become responsive to rises in progesterone levels during the secretory phase of the menstrual cycle (Critchley and Saunders, 2009).

The sex hormones acting via their specific receptors initiate a cascade of events within the endometrium which are crucial for the successful implantation of the embryo and occurs during the mid-secretory phase in a period known as the 'window of implantation' (Critchley and Saunders, 2001). As estrogen, progesterone and androgens all play vital roles within the human endometrium; it is known that any alterations in either the local hormone levels, the expression profiles of their corresponding steroid receptors and the expression of local enzymes which affect local ligand bioavailability, may all impact crucial processes within the endometrium (Critchley and Saunders, 2001). In addition to the well characterised sex steroid hormones, it is now also known that the human endometrium expresses the glucocorticoid receptor (GR) and can therefore be targeted by GCs which includes the stress hormone cortisol (Bamberger et al, 2001).

Furthermore, the GR signalling pathway regulation is controlled by the local bio-availability of the active ligand; cortisol, where the two enzymes responsible for this process are 11β-HSD1 and 11β-HSD2 (McDonald et al, 2006). More importantly, distinct patterns of 11β-HSD1 expression have been established within the human endometrium, particularly within the ESCs during the menstrual cycle (Kuroda et al, 2013).

Well-characterised infertile endometrial pathologies, such as PCOS and endometriosis have altered endometrial expression profiles of the sex steroid hormone receptors together with altered levels of the circulating sex steroid hormones during the menstrual cycle (Hulchiy et al, 2016). Additionally, the less characterised phenomenon of unexplained infertility (UI) is also now known to have altered expression levels of ER in particular, during the window of implantation (Dorostghoal et al, 2018). However, little research has been conducted on determining endometrial GR expression levels in infertile patients and whether the

expression profile of GR throughout the menstrual cycle differs to fertile patients. More importantly, the effect of an active GR signalling pathway on endometrial reproductive processes has not yet been elucidated.

In addition to the local availability of cortisol within the body, it has also been suggested that circulating systemic levels of the stress hormone may also be having an effect on health and disease. This is of interest to this study, as those suffering from infertile pathologies, particularly those suffering from PCOS and endometriosis have been associated with a list of psychopathological disturbances and a reduced quality of a life due to a manifestation of their clinical symptoms (Luisi et al, 2015; Stefanaki et al, 2015). This implies that these patients may suffer alterations in their HPA axis in response to chronic stress and therefore have altered levels of circulating cortisol, which may have a significant impact on the homeostasis of crucial processes within the body.

Even though a basic understanding of circulating levels of cortisol within the human body and the expression levels of both GR and the 11β-HSD enzymes within the human endometrium exists, a more thorough understanding is needed to identify the endometrial levels of expression throughout the menstrual cycle in not only fertile patients but also infertile patients, which has been largely ignored.

The work described in this chapter aims to characterise and assess the basal protein expression levels and localisation of GR in primary endometrial tissue isolated from fertile and infertile patients using IHC. Infertile patients were grouped according to their pathology which included ovPCOS, anPCOS, endometriosis and UI and all study groups were further grouped according to the phase of the menstrual cycle. Thus allowing us to identify whether GR expression is influenced by endometrial pathology and/or the stage of endometrial development. Secondly, this chapter also aimed to assess the circulating serum levels of the key stress hormones in the fertile and infertile patients recruited for this study, throughout the menstrual cycle and establish whether these hormones had a distinct circulating level compared to the well-characterised sex steroid hormones. Finally, whole endometrial biopsy samples isolated from fertile and infertile patients were further investigated to establish the expression of GR and 11β-HSD at the protein and mRNA level using immunoblot and qPCR analysis respectively. These samples were also grouped according to pathology and menstrual cycle phase.

3.2. Results

3.2.1. Patient cohort and demographics

One hundred and sixteen patients were recruited to participate in this study. Patients were classified in to five groups; fertile (control group) (n=24), patients suffering from unexplained infertility (UI) (n=29), patients diagnosed with endometriosis (n=29), patients diagnosed with ovulatory PCOS (ovPCOS) (n=12) and finally patients diagnosed with anovulatory PCOS (anPCOS) (n=22). Demographic data, such as age and body mass index (BMI) was collected from all patients who were recruited to the study. This data was compared between the infertile study groups (UI, endometriosis, ovPCOS and anPCOS) and the control fertile group using a parametric test (student's *t* test) (table 3.1). As mentioned previously in the materials and methods, a homogenous set of patients were identified for the study, which is represented in table 3.1. No significant differences in terms of demographic data (mean age and BMI) were observed in the patient cohorts recruited to this study.

Additionally, the serum concentration of progesterone (P_4), FSH and LH were also retrieved and are presented in table 3.1. It was found that the neither the UI patient cohort nor the endometriosis cohort had no significant difference in the concentrations of P_4. FSH or LH compared to the control fertile group. However, both PCOS cohorts had a significantly higher level of LH compared to the fertile cohort group, which altered the FSH/LH ratio significantly.

	Fertile	Unexplained infertility (UI)	Endometriosis	Ovulatory PCOS (ovPCOS)	Anovulatory PCOS (anPCOS)
Age	29.58 ± 4.12	27.1±2.4 *(P=0.440)*	28.63 ± 4.55 (p=0.727)	28.72 ± 5.06 (p=0.777)	25.1 ± 4.4 (p=0.267)
BMI (kg/m^2)	26.70 ± 4.82	28.6±2.01 *(P=0.672)*	27.54 ± 3.08 (p=0.753)	26.65 ± 3.34 (p=0.985)	28.15 ± 2.3 (p=0.591)
Progesterone day 21 (ng/ml)	31±5.28	26±4.87 *(P=0.249)*	31.27±3.89 *(P=0.894)*	27.45±11.5 *(P=0.597)*	*NA*
FSH (mUI/ml)	7.4±3.017	6.389±1.34 *(P=0.134)*	5.80±2.67 *(P=0.421)*	5.67±1.37 *(P=0.135)*	5.34±2.57 (P=0.2041)
LH (mUI/ml)	4.90±2.221	5.022±2.62 *(P=0.91)*	3.68±1.22 *(P=0.369)*	12.70±4.27 *(P=0.0004)*	15.63±4.94 *(P=0.0008)*
FSH/LH ratio	1.623±0.57	1.55±0.75 *(P=0.6441)*	1.37±0.46 *(P=0.526)*	0.48±0.151 *(P=0.0001)*	0.363±0.19 *(P=0.0005)*

Table 3.1 Table displaying patient demographic information and levels of progesterone, Follicle Stimulating Hormone (FSH) and Luteinising Hormone (LH) obtained by our collaborators at the ABMUHB biochemistry department. Values are averaged ± SD. Data was analysed by the ABMUHB biochemistry department using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

The expression of GR was consequently evaluated in formalin fixed paraffin embedded (FFPE) samples by immunohistochemistry (IHC) as described in materials and methods (section 2.2). IHC slides were scored by three individual observers, according to protein intensity and distribution of staining. All data was found to be non-normally distributed after analysis using the Kolmogorov-Smirnov test, which is a test for normality. Therefore, IHC data was analysed for statistically significant differences using the non-parametric Kruskall-Wallis test followed by the Mann-Whitney U test, to identify significant differences between patient cohorts.

3.2.2. Expression of the glucocorticoid receptor (GR) in endometrial biopsies isolated from fertile patients during the menstrual cycle

The expression of GR was analysed in the endometrial biopsy samples isolated from fertile patients, which has been embedded in FFPE as previously mentioned. The method of IHC was described fully in the materials and methods (section 2.2), but briefly, an anti-GR antibody specific to GRα and β was used for staining. Initially, GR expression was analysed in biopsy samples isolated from fertile patients in both the glandular and stromal region during the proliferative phase of the menstrual cycle.

It was found that there were no significant differences in the expression levels of GR in the stromal region compared to the glandular region of the endometrium in the fertile patients during the proliferative phase of the menstrual cycle ($n=10$) ($p \geq 0.05$) (figure 3.1 and figure 3.3).

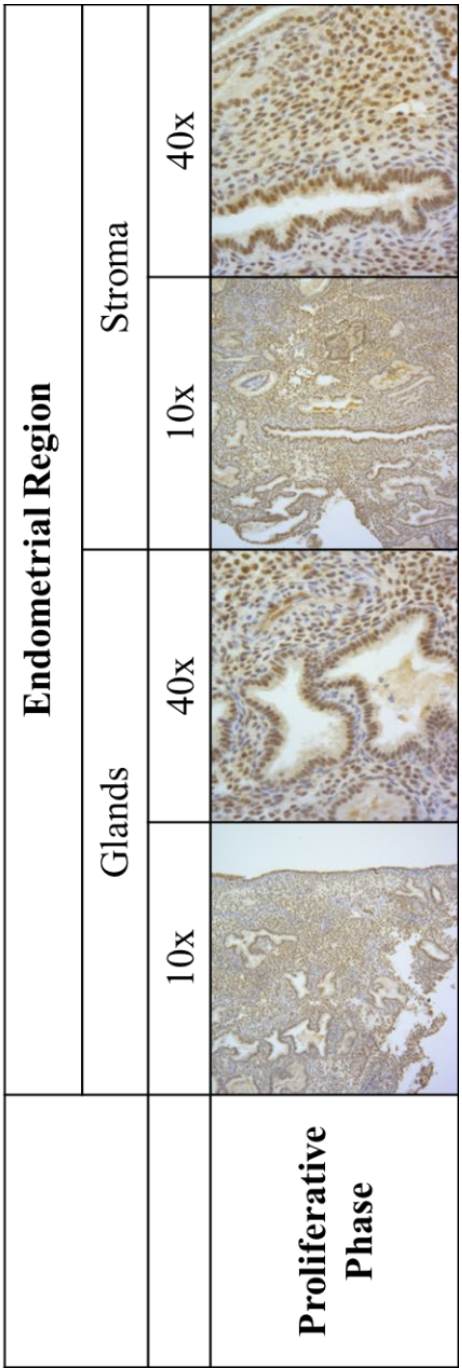


Figure 3.1 The IHC staining showing the expression of the glucocorticoid receptor (GR) in the glandular and stromal region in fertile patients during the proliferative phase of the menstrual cycle. Biopsy samples obtained from fertile patients during the proliferative phase of the menstrual cycle were stained with an anti-rabbit anti-GR antibody and analysed using IHC. GR expression was identified in the glands and the stroma. Representative IHC images are shown for each group, at both x10 and x40 magnification. IHC samples were scored blind in triplicate by three independent observers.

Conversely, it was found that there was a significant difference in the expression levels of GR in the glandular and stromal region in the fertile patients during the secretory phase of the menstrual cycle (n=14) ($p \leq 0.001$) (figure 3.2 and figure 3.3).

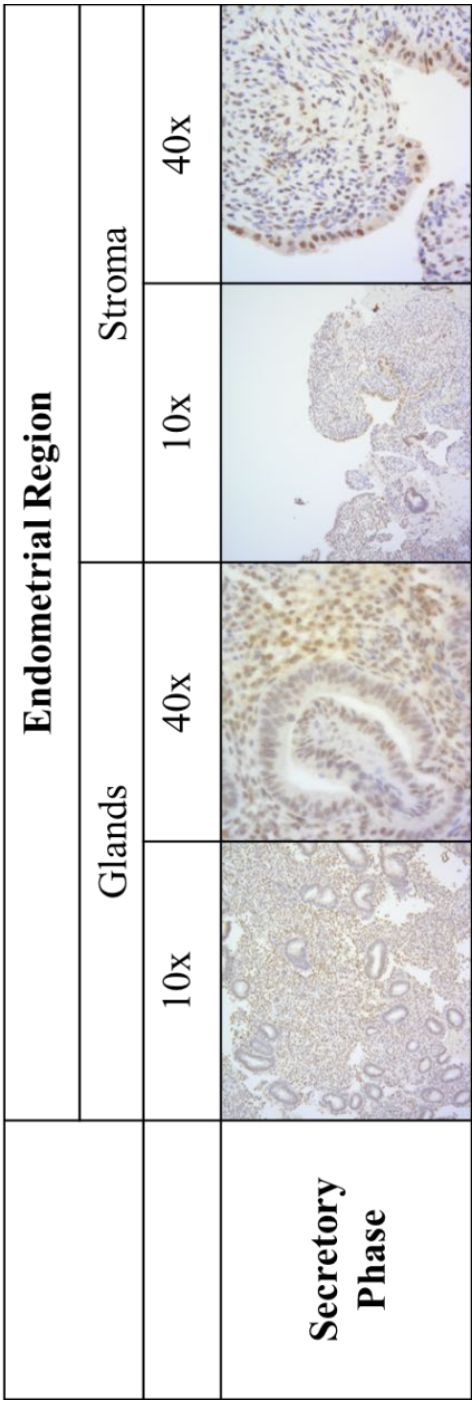


Figure 3.2 The IHC staining showing the expression of the glucocorticoid receptor (GR) in the glandular and stromal region in fertile patients during the secretory phase of the menstrual cycle. Biopsy samples obtained from fertile patients during the secretory phase of the menstrual cycle were stained with an anti-rabbit anti-GR antibody and analysed using IHC. GR expression was identified in the glands and the stroma. Representative IHC images are shown for each group, at both x10 and x40 magnification. IHC samples were scored blind in triplicate by three independent observers.

The data obtained for GR expression during the proliferative and secretory phase of the menstrual cycle was compared. It was found that the glandular region of the endometrium had a significantly lower expression of GR in terms of IHC staining during the secretory phase of the menstrual cycle compared to the proliferative phase ($p \leq 0.01$) (figure 3.3). Additionally, it was also found that the stromal region of the endometrium also had a significantly lower expression of GR during the secretory phase compared to the proliferative phase of the menstrual cycle ($p \leq 0.001$) (figure 3.3). It is worth mentioning that the glandular expression of GR has a uniform distribution across all IHC images analysed and the differences observed are mainly due to differences in intensity of the staining. Whereas, in the case of the stromal region, reductions in both the intensity and the distribution of GR staining were observed between the proliferative and secretory phases of the menstrual cycle, where a significant reduction in the amount of cells stained were observed in the secretory phase.

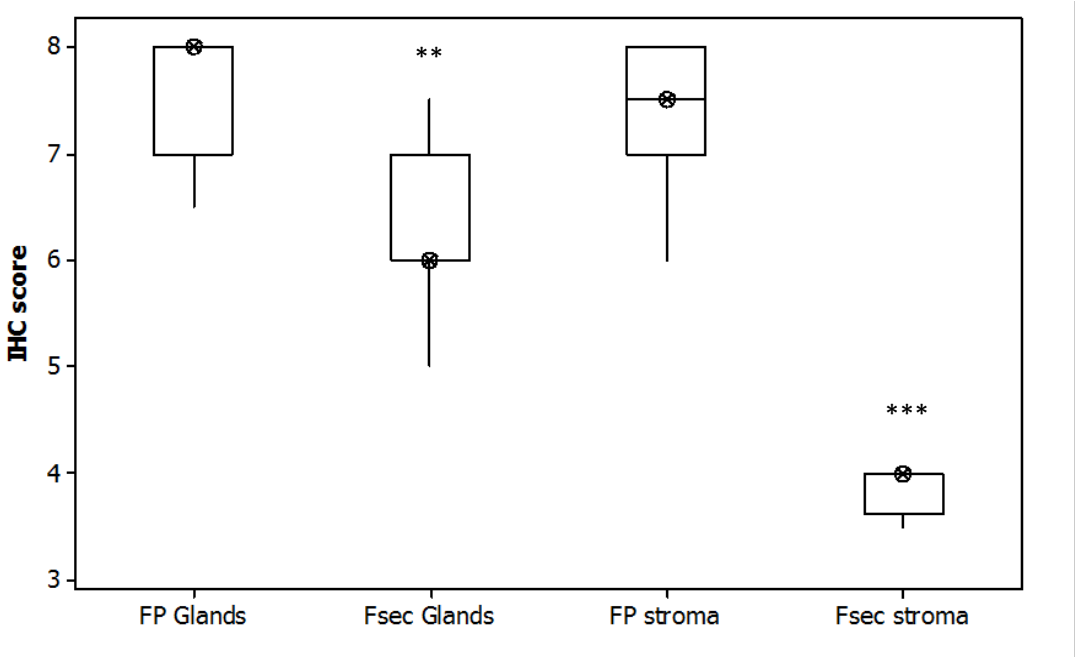


Figure 3.3 The expression of the glucocorticoid receptor (GR) in the glandular and stromal region in fertile patients during the secretory and proliferative phase of the menstrual cycle. Box plot shows GR protein levels in endometrial biopsy specimens obtained from fertile patients during the proliferative phase (n=10) and secretory phase (n=14) of the menstrual cycle by IHC. IHC samples were scored blind in triplicate by three independent observers. Values given are mean H-score and bars indicate ± SD. Statistical analysis was performed using the Kruskall-Wallis test followed by the Mann-Whitney test. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ are considered significant.

3.2.3. Steroid hormone levels in the serum isolated from fertile patients during the menstrual cycle

After identifying the levels of GR expression in the endometrial biopsy samples isolated from fertile patients, it was of interest to investigate the levels of circulating key hormones in the serum isolated from these patients. Blood samples were isolated from patients enrolled on this study (described in materials and methods, section 2.8.2). Briefly, the blood samples were centrifuged and the serum was collected to analyse using commercially available ELISA kits. Identification of the circulating levels of these hormones would allow us to correlate hormone levels to the expression of GR in the endometrium of fertile patients during the menstrual cycle.

Initially, the serum samples obtained from the fertile patients enrolled on this study were analysed for the concentration of circulating cortisol.

Interestingly, it was found that fertile patients who were in the secretory phase of the menstrual cycle had a significantly lower concentration of circulating cortisol compared to patients who were in the proliferative phase of their cycle ($p \leq 0.05$) (figure 3.4). Furthermore, it was apparent that during the menses phase of the cycle, fertile patients have a significantly higher level of circulating cortisol compared to those in the secretory phase ($p \leq 0.05$). No significant difference in the serum levels of circulating cortisol was observed between the proliferative and menses phase (figure 3.4).

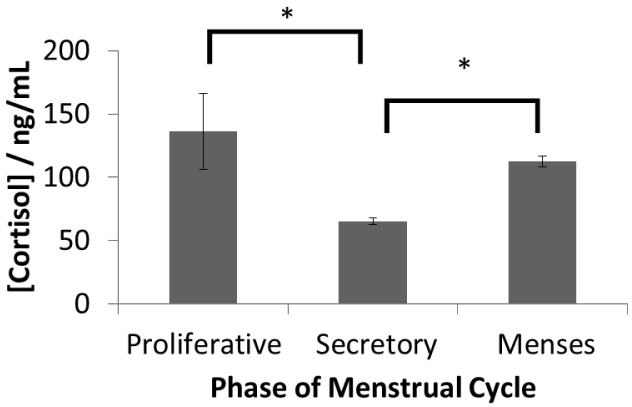


Figure 3.4 The concentration of cortisol (compound F) in the serum isolated from fertile patients during the proliferative, secretory and menses phase of the menstrual cycle. Blood samples from the fertile patient cohort at various stages of their menstrual cycle (proliferative n=8, secretory n=8 and menses n=4) were centrifuged and the serum obtained was analysed for the levels of circulating cortisol using an ELISA. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ are considered significant.

In addition to cortisol, the circulating levels of cortisone were also analysed. Converse results were obtained, where it was observed that fertile patients in the secretory phase of their menstrual cycle had a higher level of circulating cortisone compared to those in the proliferative phase ($p \leq 0.05$) (figure 3.5). Furthermore, it was found that patients during the menses phase of their cycle had a significantly lower circulating level of cortisone compared to those in the secretory phase ($p \leq 0.05$) (figure 3.5). This suggests that there is an active interconversion of cortisone and cortisol in the fertile patients during the distinct phases of the menstrual cycle. As seen in the cortisol results, no significant difference in the serum levels of circulating cortisone were observed between the proliferative and menses phase of the menstrual cycle (figure 3.5).

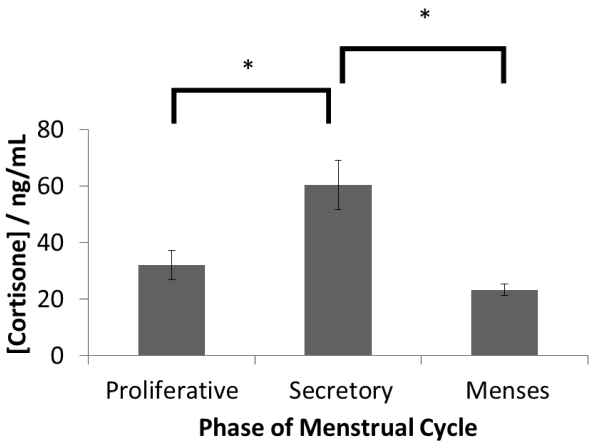


Figure 3.5 The concentration of cortisone (compound E) in the serum isolated from fertile patients during the proliferative, secretory and menses phase of the menstrual cycle. Blood samples from the fertile patient cohort at various stages of their menstrual cycle (proliferative n=8, secretory n=8 and menses n=4) were centrifuged and the serum obtained was analysed for the levels of circulating cortisone using an ELISA. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ are considered significant.

In addition to the active and inactive stress hormones, it was also deemed necessary to investigate the levels of the common sex steroid hormones, which play a crucial role during the menstrual cycle, such as estradiol, progesterone and androgens, to see whether the circulating levels of these hormones could be correlated with GR expression levels and the circulating levels of the stress hormones.

It was found using ELISA analysis, that circulating estradiol levels in the serum of fertile patients had a similar pattern to cortisone levels during the menstrual cycle (figure 3.6). It was found that during the secretory phase of the menstrual cycle there is a higher circulating

level of estradiol compared to both the proliferative phase ($p \leq 0.05$) and the menses phase ($p \leq 0.001$) of the menstrual cycle (figure 3.6). No significant differences in estradiol levels were observed between the proliferative and menses phase, as observed in the previous ELISA results for cortisone and cortisol. The results obtained to determine the circulating levels of estradiol in this cohort of patients contradicts published data to date, as it is expected that estradiol levels normally increase during the proliferative phase of the menstrual cycle. However, these results are specific to the cohort of patients recruited to this study and may be impacted by sampling time and sample size. To investigate this further in future studies, a larger sample size of patients could be recruited or repeat sampling and testing could be carried out to address this limitation.

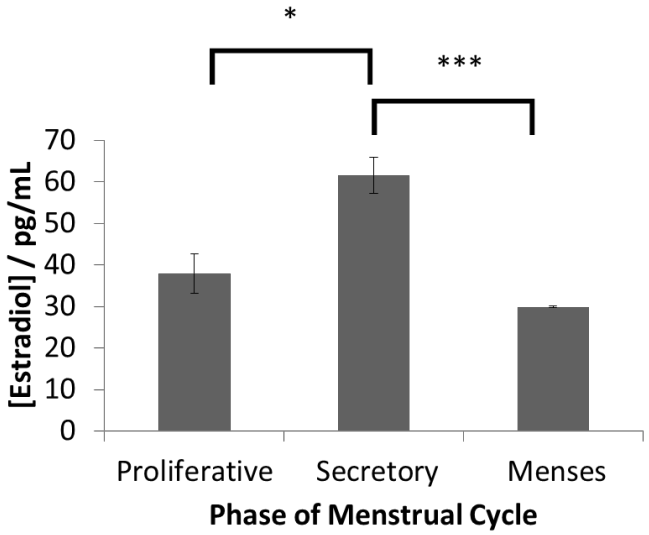


Figure 3.6 The concentration of estradiol (E_2) in the serum isolated from fertile patients during the proliferative, secretory and menses phase of the menstrual cycle. Blood samples from the fertile patient cohort at various stages of their menstrual cycle (proliferative n=8, secretory n=8 and menses n=4) were centrifuged and the serum obtained was analysed for the levels of circulating estradiol using an ELISA. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ are considered significant.

Furthermore, this similar pattern of results was reciprocated in terms of circulating progesterone levels. It was found that the highest levels of circulating progesterone were found during the secretory phase of the menstrual cycle in the fertile patients (figure 3.7). This high level of circulating progesterone was significantly higher than that seen during the proliferative phase of the menstrual cycle ($p \leq 0.05$) (figure 3.7). No significant differences in the concentration of progesterone were observed between the proliferative and menses phase of the menstrual cycle or between the secretory and menses phase (figure 3.7).

Finally, the circulating level of androgens present in the serum which was isolated from fertile patients was investigated during the proliferative phase only, as PCOS patients are not able to enter the secretory phase of the menstrual cycle and therefore cannot be compared to results obtained for the control (fertile patients). The circulating levels of total testosterone (total T), free testosterone (free T), free androgen index, sex hormone binding globulin (SHBG), androstenedione (A) and dehydroepiandrosterone (DHEAS) were analysed and are presented in table 3.2.

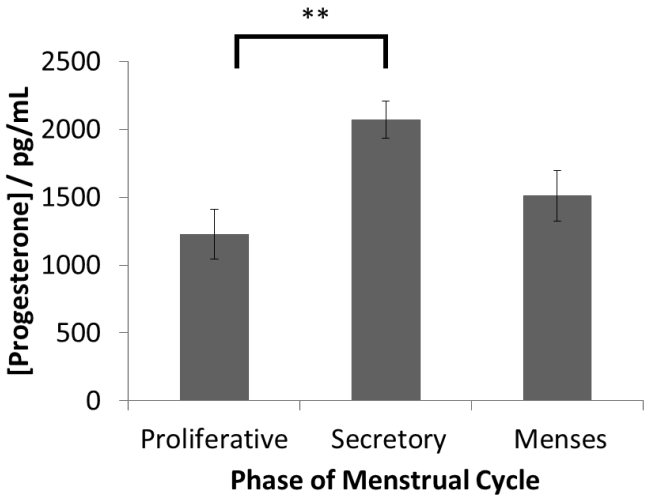


Figure 3.7 The concentration of progesterone (P_4) in the serum isolated from fertile patients during the proliferative, secretory and menses phase of the menstrual cycle. Blood samples from the fertile patient cohort at various stages of their menstrual cycle (proliferative n=8, secretory n=8 and menses n=4) were centrifuged and the serum obtained was analysed for the levels of circulating progesterone using an ELISA. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

	Fertile
Total T (nmol/liter)	0.76±0.472
Free T (pmol/liter)	13.13±6.12
Free androgen index	4.10±0.81
SHBG (nmol/liter)	42.60±5.18
A (nmol/liter)	3.43±0.23
DHEAS (µmol/liter)	5.07±0.76

Table 3.2 The concentration of the various androgens in the serum isolated from fertile patients during the proliferative phase of the menstrual cycle. Blood samples from the fertile patient cohort at the proliferative phase of the menstrual cycle (n=10) were centrifuged and the serum obtained was analysed for the levels of circulating androgens using an ELISA and was carried out by our collaborators at the ABMUHB biochemistry department. Total T = total testosterone, Free T = Free testosterone, SHBG = sex hormone binding globulin, A = androstenedione and DHEAS = dehydroepiandrosterone. Values are average ± SD. Data was analysed by ABMUHB biochemistry department using an ANOVA test, followed by a student's t-test.

3.2.4. Basal expression of the glucocorticoid receptor (GR) and 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes in whole biopsy samples isolated from fertile patients during the menstrual cycle

The IHC data presented in this chapter has so far indicated a specific expression pattern in GR which is dependant not only on the endometrial location, but also dependant on the phase of the menstrual cycle. These results were further investigated using qPCR analysis to determine the basal expression levels of GR and the 11β-hydroxysteroid dehydrogenase (11β-HSD) enzyme. Whole biopsies were isolated from fertile patients and underwent tissue homogenisation followed by DNA and protein extraction.

Contradictory to results obtained using IHC analysis, qPCR analysis revealed that the relative gene expression of GR significantly increased during the secretory phase of the menstrual cycle ($p \leq 0.05$) and consequently reduced its expression during the menses phase of the cycle. This low expression of GR was maintained throughout the proliferative phase of the cycle in the fertile patients (figure 3.8). Interestingly, the reverse pattern was obtained for the circulating levels of serum cortisol (figure 3.4). This was also seen when investigating the relative gene expression levels of 11β-HSD1, where, as seen for GR, the secretory phase of the menstrual cycle resulted in the highest expression of 11β-HSD1 compared to both the proliferative and menses phase of the cycle (not significant) (figure 3.8).

Following qPCR analysis, protein extracted from whole biopsy samples which were isolated from fertile patients were used for immunoblot analysis. The results obtained from the immunoblot analysis reciprocated the results obtained at the gene level.

It was found that the basal GR protein expression of both α and β isoforms was at its highest during the secretory phase of the menstrual cycle in the fertile patients (figure 3.9 A, B and C). GRα and GRβ protein expression was significantly higher in the secretory phase of the cycle compared to the proliferative phase ($p \leq 0.01$ and $p \leq 0.05$ respectively). This level then dropped significantly during the menses phase ($p \leq 0.05$ and ≤ 0.01), where the protein levels of both GR isoforms remained low during the proliferative phase.

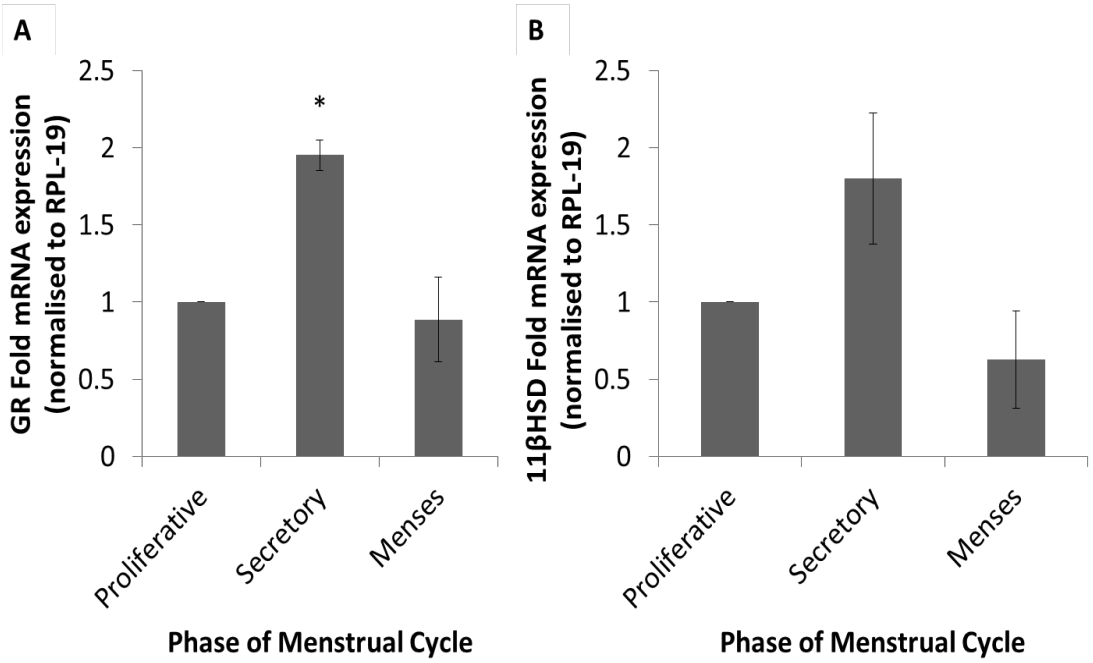


Figure 3.8. Basal mRNA expression levels of GR (A) and 11βHSD (B) in the proliferative, secretory and menses phase of the menstrual cycle of fertile patients. Graphs show GR mRNA levels in whole tissue biopsy specimens in the proliferative, secretory and menses phase of the menstrual cycle by qPCR. Values shown are the mean fold expression of mRNA from triplicate values per sample relative to the proliferative phase of the menstrual cycle and normalised to the housekeeper gene; RPL-19. Fertile patients were grouped by phase of the menstrual cycle; proliferative phase (n=3), secretory phase (n=3) and menses phase (n=3). Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

Comparable results were obtained for the basal protein levels of 11β-HSD in whole biopsies isolated from fertile patients, where the highest level of protein expression was observed during the secretory phase of the cycle, which then reduced during the menses phase and remained low throughout the proliferative phase (figure 3.9 A and D). Even though this pattern was observed for 11β-HSD protein expression, the differences in the protein expression levels seen in each phase of the cycle were not deemed to be statistically significant.

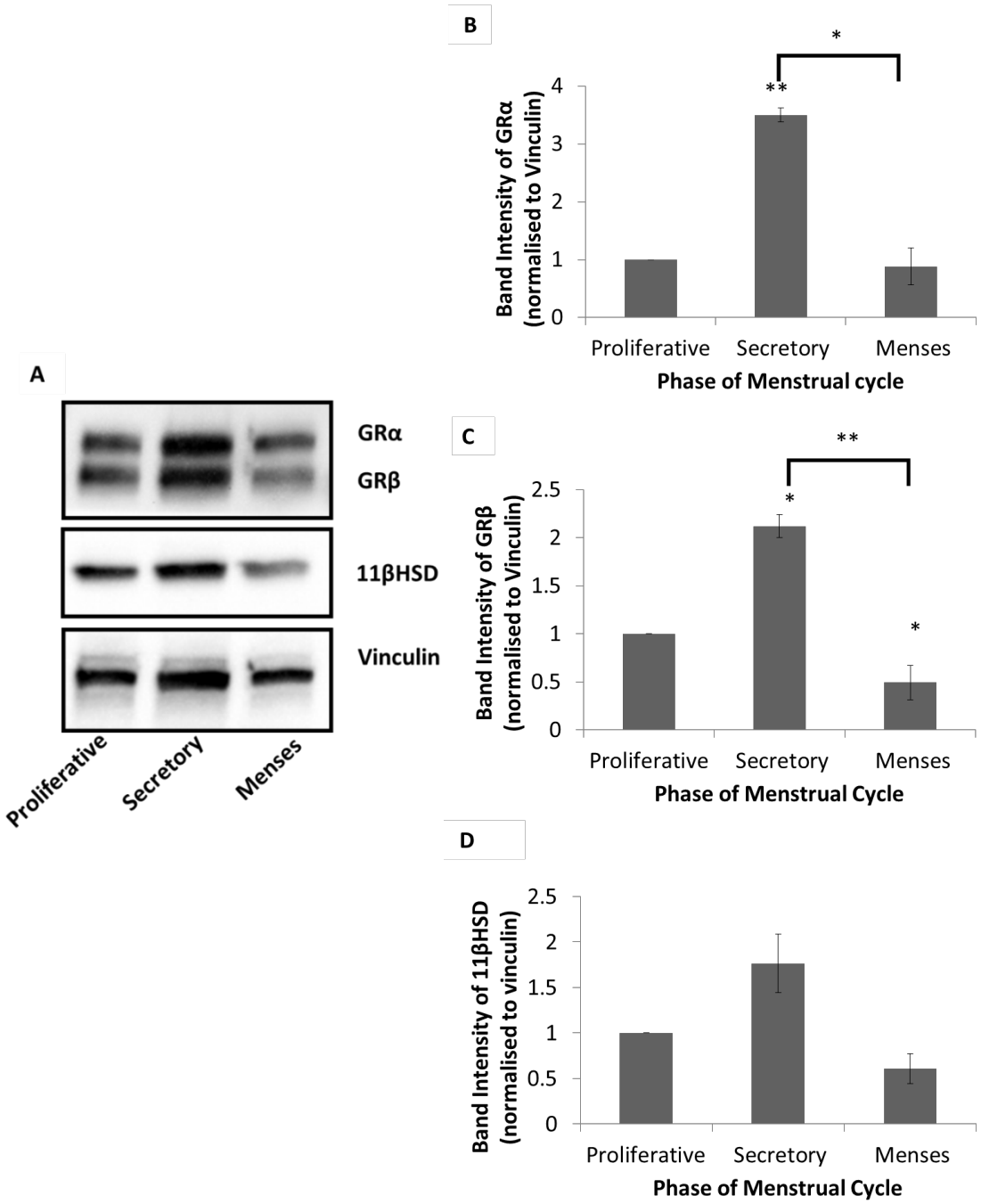


Figure 3.9. Basal protein expression levels of GRα (A and B), GRβ (A and C) and 11βHSD (A and D) in the proliferative, secretory and menses phase of the menstrual cycle of fertile patients. Figure shows immunoblot images of GRα (95 kDa), GRβ (90 kDa), 11β-HSD (32 kDa) and the housekeeper protein; vinculin (124 kDa) in whole tissue biopsy specimens in the proliferative, secretory and menses phase of the menstrual cycle (A). Graphs show the mean band intensity values from triplicate values normalised to the housekeeper protein; vinculin. The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the house keeper protein; vinculin. Band intensity is displayed as the fold expression relative to the proliferative phase of the menstrual cycle and normalised to the respective vinculin samples. Fertile patients were grouped by phase of the menstrual cycle; proliferative phase (n=3), secretory phase (n=3) and menses phase (n=3). Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

3.2.5. Expression of the glucocorticoid receptor (GR) in endometrial biopsies isolated from infertile patients during the menstrual cycle

Following the analysis of the whole biopsies isolated from fertile patients, it was decided to also investigate the expression of GR in endometrial biopsy samples isolated from infertile patients. This included the following patient cohorts; anovulatory PCOS (anPCOS), ovulatory PCOS (ovPCOS), endometriosis and unexplained infertility (UI) which were compared to the results obtained for the fertile control patients. The biopsies isolated from the infertile patients were as previously mentioned, embedded in FFPE. The expression of GR was compared in the biopsy samples in both the glandular and stromal region during the proliferative phase of the menstrual cycle.

It was found that there was a significant difference in the expression levels of GR in the glandular region of the endometrium in the infertile patient groups compared to fertile patients during the proliferative phase of the menstrual cycle (figure 3.10). IHC analysis revealed that during the proliferative phase, the glandular region expression of GR is significantly lower in patients suffering from anPCOS, ovPCOS, endometriosis and UI ($p \leq 0.05$, $p \leq 0.05$, $p \leq 0.05$ and $p \leq 0.01$ respectively) (figure 3.10).

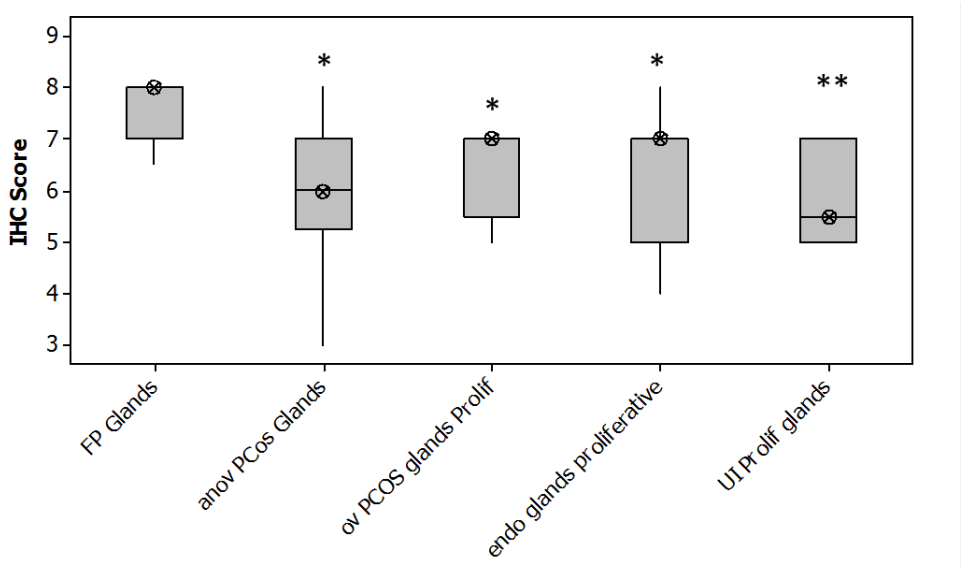


Figure 3.10 The expression of the glucocorticoid receptor (GR) in the glandular region of the endometrium in patients suffering from anovulatory PCOS (anPCOS), ovulatory PCOS (ovPCOS), endometriosis and unexplained infertility (UI) and compared to fertile patients during the proliferative phase of the menstrual cycle. Box plot shows GR protein levels in endometrial biopsy specimens obtained from infertile (anPCOS n=22, ovPCOS n=6, endometriosis n=14 and UI n=13) and fertile patients (n=10) during the proliferative phase of the menstrual cycle by IHC. IHC samples were scored blind in triplicate by three independent observers. Values given are mean H-score and bars indicate ± SD. Statistical analysis was performed using the Kruskall-Wallis test followed by the Mann-Whitney test. *$p \leq 0.05$, **$p \leq 0.01$ are considered significant.

Similarly, it was found that there was also a significant difference in the expression levels of GR in the stromal region of the endometrium in the infertile patient groups compared to fertile patients during the proliferative phase of the menstrual cycle (figure 3.11). IHC analysis revealed that during the proliferative phase, the stromal region expression of GR is significantly lowers in patients suffering from anPCOS, ovPCOS, endometriosis and UI ($p \leq 0.01$) (figure 3.11).

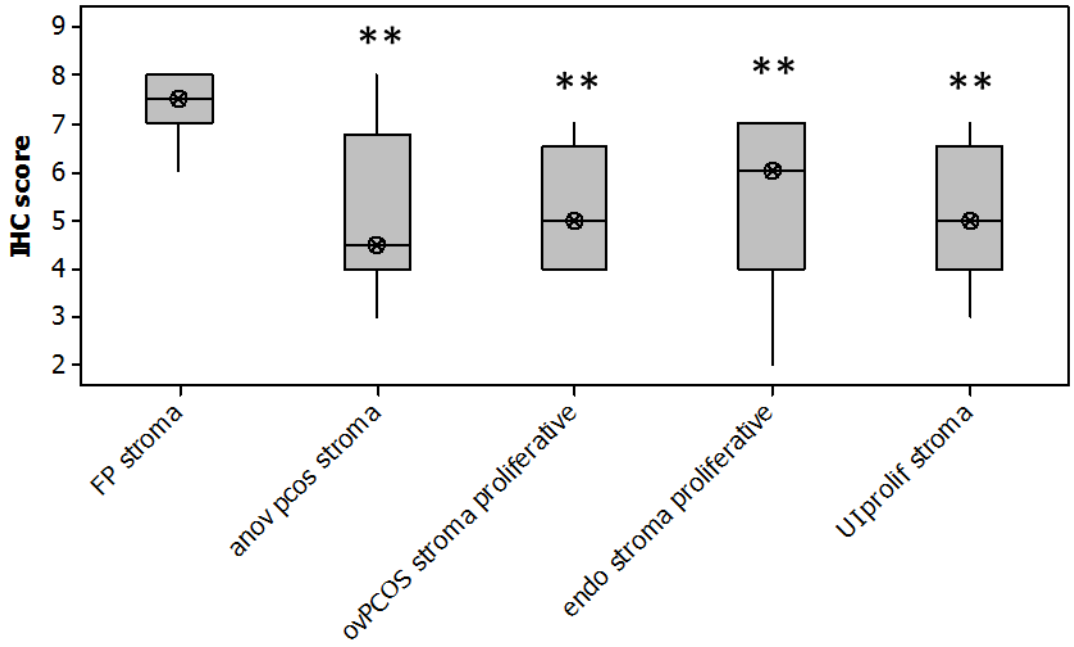


Figure 3.11 The expression of the glucocorticoid receptor (GR) in the stromal region of the endometrium in patients suffering from anovulatory PCOS (anPCOS), ovulatory PCOS (ovPCOS), endometriosis and unexplained infertility (UI) and compared to fertile patients during the proliferative phase of the menstrual cycle. Box plot shows GR protein levels in endometrial biopsy specimens obtained from infertile (anPCOS n=22, ovPCOS n=6, endometriosis n=14 and UI n=13) and fertile patients (n=10) during the proliferative phase of the menstrual cycle by IHC. IHC samples were scored blind in triplicate by three independent observers. Values given are mean H-score and bars indicate ± SD. Statistical analysis was performed using the Kruskall-Wallis test followed by the Mann-Whitney test. *$p \leq 0.05$, **$p \leq 0.01$ are considered significant.

In addition to investigating the GR expression levels during the proliferative phase of the cycle, biopsies were also retrieved from patients suffering from ovPCOS, endometriosis and UI during the secretory phase of the menstrual cycle. Patients suffering from anPCOS were not able to be included in this analysis due to anPCOS patients being unable to progress to the secretory phase of the menstrual cycle. It was found using IHC analysis that there was no significant difference in the expression levels of GR in the glandular region of the endometrium between the infertile patient groups under investigation and the fertile patients during the secretory phase of the menstrual cycle (figure 3.12).

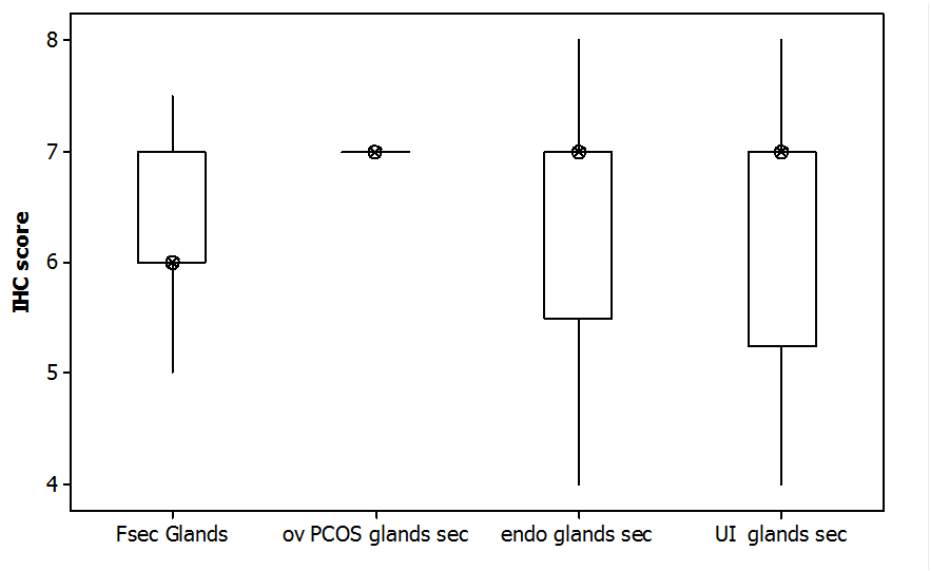


Figure 3.12 The expression of the glucocorticoid receptor (GR) in the glandular region of the endometrium in patients suffering from ovulatory PCOS (ovPCOS), endometriosis and unexplained infertility (UI) and compared to fertile patients during the secretory phase of the menstrual cycle. Box plot shows GR protein levels in endometrial biopsy specimens obtained from infertile (ovPCOS n=6, endometriosis n=15 and UI n=16) and fertile patients (n=14) during the secretory phase of the menstrual cycle by IHC. IHC samples were scored blind in triplicate by three independent observers. Values given are mean H-score and bars indicate ± SD. Statistical analysis was performed using the Kruskall-Wallis test followed by the Mann-Whitney test. *p≤0.05, **p≤0.01 are considered significant.

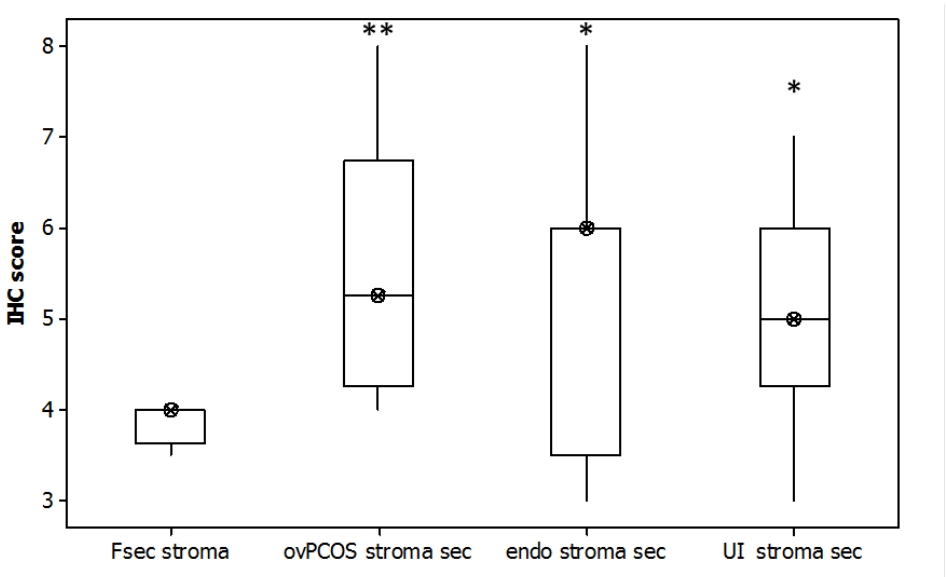


Figure 3.13 The expression of the glucocorticoid receptor (GR) in the stromal region of the endometrium in patients suffering from ovulatory PCOS (ovPCOS), endometriosis and unexplained infertility (UI) and compared to fertile patients during the secretory phase of the menstrual cycle. Box plot shows GR protein levels in endometrial biopsy specimens obtained from infertile (ovPCOS n=6, endometriosis n=15 and UI n=16) and fertile patients (n=14) during the secretory phase of the menstrual cycle by IHC. IHC samples were scored blind in triplicate by three independent observers. Values given are mean H-score and bars indicate ± SD. Statistical analysis was performed using the Kruskall-Wallis test followed by the Mann-Whitney test. *p≤0.05, **p≤0.01 are considered significant.

Conversely, it was found that during the secretory phase of the menstrual cycle there is an increase in GR expression in the stromal region of the endometrium in the patients suffering from ovPCOS ($p \leq 0.01$), endometriosis ($p \leq 0.05$) and UI ($p \leq 0.05$) (figure 3.13).

3.2.6. Steroid hormone levels in the serum isolated from infertile patients during the menstrual cycle

After identifying the levels of GR expression in the endometrial biopsy samples isolated from infertile patients, it was decided to study the levels of the circulating levels of key hormones in the serum isolated from these patients. Isolated blood samples were processed to retrieve serum only and were analysed for hormone levels using commercially available ELISA kits. The identification of the circulating levels of these hormones would allow for the correlation of hormone concentration and the expression of GR within the endometrium of infertile patients during the menstrual cycle.

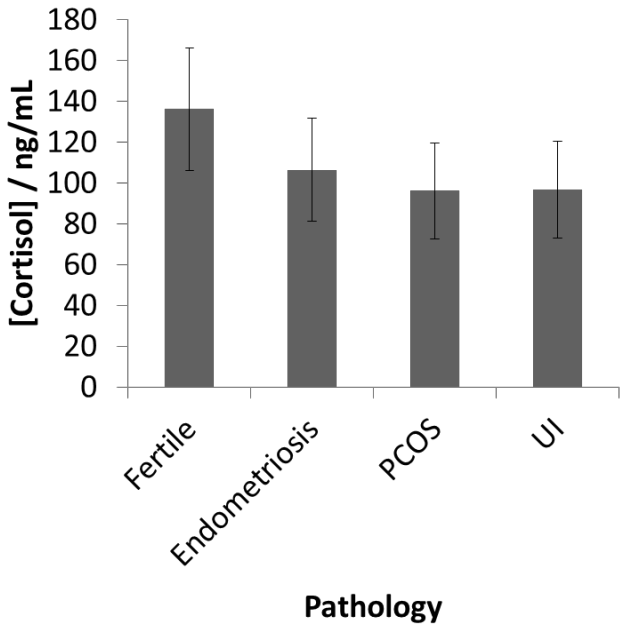


Figure 3.14 The concentration of cortisol (compound F) in the serum isolated from fertile and infertile patients during the proliferative phase of the menstrual cycle. Blood samples from the fertile (n=8) and infertile patient cohorts during the proliferative phase of their menstrual cycle (endometriosis n=8, PCOS n=6 and UI n=8) were centrifuged and the serum obtained was analysed for the levels of circulating cortisol using an ELISA. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ are considered significant.

Initially, the serum samples obtained from the infertile patient cohorts enrolled on this study were analysed for the concentration of circulating cortisol and compared to fertile control patients.

Interestingly, it was found that even though there seems to be a decrease in the levels of circulating cortisol during the proliferative phase of the cycle in patients suffering from PCOS, endometriosis and UI – this difference was not considered to be statistically significant (figure 3.14).

In addition to cortisol, the circulating levels of cortisone were also analysed, and similar to the results obtained for the fertile patients, contrary results were obtained. It was found that the infertile patient groups have an increase in circulating levels of cortisone overall during the proliferative phase of the menstrual cycle (figure 3.15). However, it was found that there was only a statistically significant increase in the concentration of cortisone in the UI cohort compared to the fertile patients ($p \leq 0.05$).

The levels of circulating sex hormones were also investigated in the infertile patients to identify if the levels of these common hormones such as estradiol, progesterone and androgens can be correlated with GR expression levels and the circulating levels of the stress hormones.

It was found using ELISA analysis that the circulating serum levels of estradiol varies in the patient study groups during the proliferative phase of the menstrual cycle (figure 3.16). It was found that PCOS patients have comparable circulating estradiol levels to fertile patients, and even though endometriosis and UI patients appear to have a slightly higher level of estradiol present in their serum, this was not deemed to be significant. However, after statistical analysis, it was found that the circulating levels of estradiol in endometriosis patients are significantly higher than both PCOS patients ($p \leq 0.01$) and UI patients ($p \leq 0.05$) (figure 3.16). This pattern of results differs greatly to those obtained for both circulating levels of cortisol and cortisone as discussed previously.

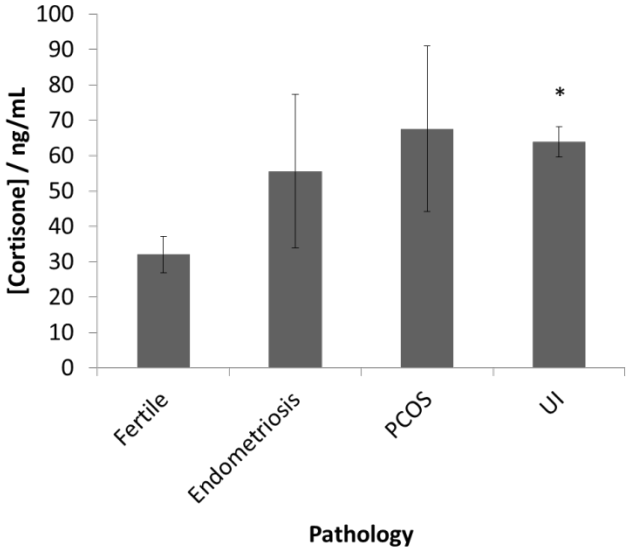


Figure 3.15 The concentration of cortisone (compound E) in the serum isolated from fertile and infertile patients during the proliferative phase of the menstrual cycle. Blood samples from the fertile (n=8) and infertile patient cohorts during the proliferative phase of their menstrual cycle (endometriosis n=8, PCOS n=6 and UI n=8) were centrifuged and the serum obtained was analysed for the levels of circulating cortisone using an ELISA. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

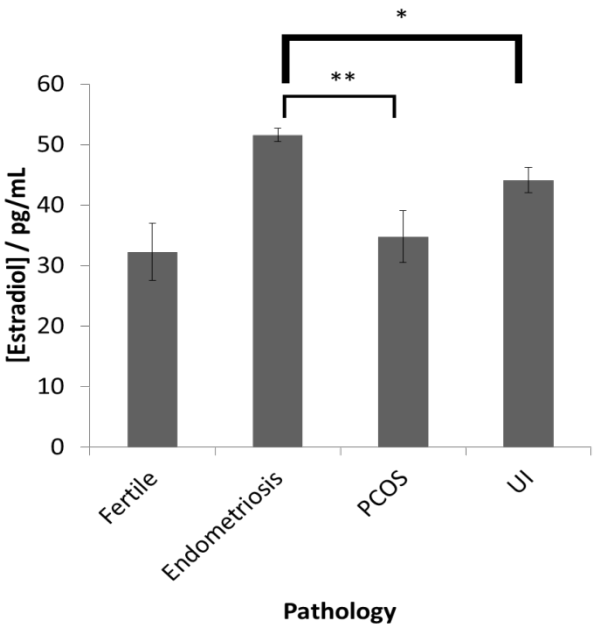


Figure 3.16 The concentration of estradiol (E_2) in the serum isolated from fertile and infertile patients during the proliferative phase of the menstrual cycle. Blood samples from the fertile (n=8) and infertile patient cohorts during the proliferative phase of their menstrual cycle (endometriosis n=8, PCOS n=6 and UI n=8) were centrifuged and the serum obtained was analysed for the levels of circulating estradiol using an ELISA. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

The serum was then used to analyse the levels of circulating progesterone in the infertile patient cohorts and compared to fertile patients during the proliferative phase of the menstrual cycle.

It was found that the levels of progesterone in the infertile PCOS and endometriosis patients followed a similar pattern to the levels of cortisone seen during the proliferative phase of the menstrual cycle. ELISA analysis revealed that on average, the circulating levels of progesterone in endometriosis and PCOS patients are higher than those observed in the fertile patients during the proliferative phase (figure 3.17), however this difference in concentration is not significant. On the other hand, levels of progesterone in UI patients seems to be comparable to levels observed in the fertile patients (figure 3.17).

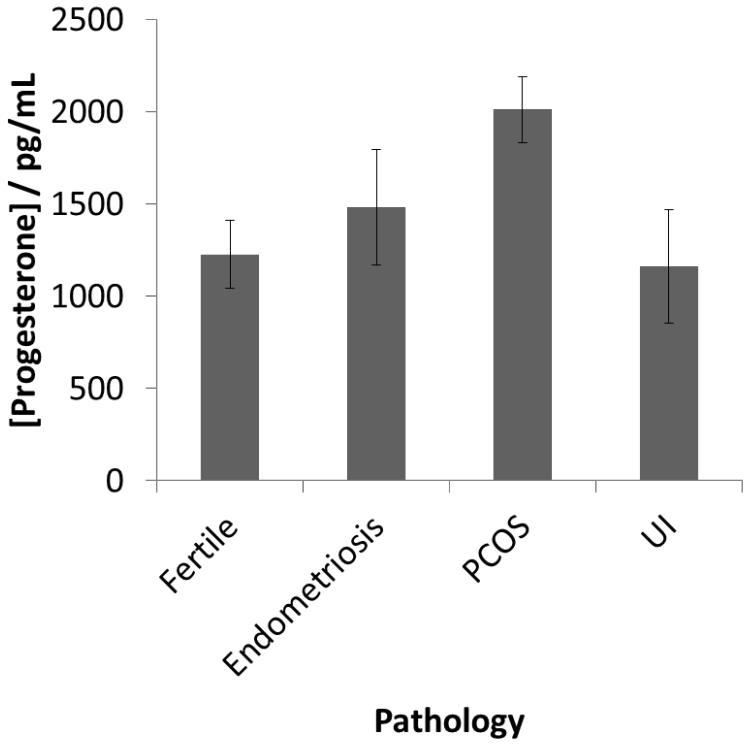


Figure 3.17 The concentration of progesterone (P_4) in the serum isolated from fertile and infertile patients during the proliferative phase of the menstrual cycle. Blood samples from the fertile (n=8) and infertile patient cohorts during the proliferative phase of their menstrual cycle (endometriosis n=8, PCOS n=6 and UI n=8) were centrifuged and the serum obtained was analysed for the levels of circulating progesterone using an ELISA. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

Lastly, the circulating levels of androgens present in the serum isolated from infertile patients suffering from ovPCOS and anPCOS was investigated during the proliferative phase of the menstrual cycle and compared to results obtained for the control fertile patients.

It was found that anPCOS patients only had a significantly higher level of total testosterone, free testosterone and SHBG present in their serum compared to fertile patients ($p \leq 0.001$, $p \leq 0.01$ and $p \leq 0.05$ respectively) (table 3.3). Furthermore, both patients suffering from ovPCOS and anPCOS had a significantly higher free androgen index, androstenedione and DHEAS compared to fertile patients during the proliferative phase of the menstrual cycle ($p \leq 0.05$ and $p \leq 0.01$, $p \leq 0.01$ and $p \leq 0.001$, $p \leq 0.05$ and $p \leq 0.01$) (table 3.3). Circulating androgen levels in endometriosis patients were not collected.

	Fertile	Ovulatory PCOS	Anovulatory PCOS
Total T (nmol/liter)	0.76±0.472	1.72±0.313 *(P=0.1)*	2.94±0.108 *(P=0.001)*
Free T (pmol/liter)	13.13±6.12	18.5±5.7 (P=0.08)	37.15 ± 10.6 (P=0.002)
Free androgen index	4.10±0.81	5.6±0.39 (P=0.05)	8.12±1.16 (P=0.003)
SHBG (nmol/liter)	42.60±5.18	34.51±2.97 (P=0.8)	27.92± 10.5 (P=0.05)
A (nmol/liter)	3.43±0.23	7.70±1.6 (P=0.006)	8.72±1.1 (P=0.0001)
DHEAS (µmol/liter)	5.07±0.76	7.26±0.61 (P=0.03)	7.560±0.32 (P=0.01)

Table 3.3 The concentration of the various androgens in the serum isolated from fertile and infertile patients during the proliferative phase of the menstrual cycle. Blood samples from the fertile (n=10) and infertile patient cohorts during the proliferative phase of their menstrual cycle (ovPCOS n=6 and anPCOS n=22) were centrifuged and the serum obtained was analysed for the levels of circulating progesterone using an ELISA and was carried out by our collaborators at the ABMUHB biochemistry department. Total T = total testosterone, Free T = Free testosterone, SHBG = sex hormone binding globulin, A = androstenedione and DHEAS = dehydroepiandrosterone. Values are average ± SD. Data was analysed by ABMUHB biochemistry department using an ANOVA test, followed by student t-test. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ are considered significant.

The previous ELISA experiments were consequently repeated using serum obtained from patients in the secretory phase of their menstrual cycle. As previously mentioned, anPCOS patients were not able to be included in this part of the study due to their inability to progress to the secretory phase.

Interestingly, several converse results were obtained when compared to those seen during the proliferative phase of the menstrual cycle. It was found that during the secretory phase of the menstrual cycle, fertile and endometriosis patients have similar levels of circulating cortisol (figure 3.18). However, both ovPCOS and UI patients have significant higher levels of circulating cortisol when compared to fertile patients during the secretory phase ($p \leq 0.01$ and $p \leq 0.05$ respectively) (figure 3.18).

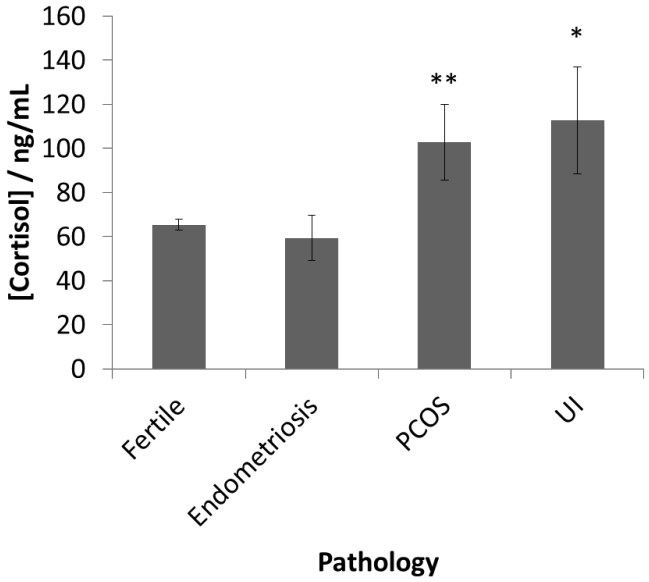


Figure 3.18 The concentration of cortisol (compound F) in the serum isolated from fertile and infertile patients during the secretory phase of the menstrual cycle. Blood samples from the fertile (n=8) and infertile patient cohorts during the secretory phase of their menstrual cycle (endometriosis n=8, PCOS n=8 and UI n=6) were centrifuged and the serum obtained was analysed for the levels of circulating cortisol using an ELISA. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ are considered significant.

In addition to cortisol, the circulating levels of cortisone were also analysed in serum isolated from patients suffering from endometriosis, ovPCOS and UI and compared to fertile patients. Conversely, to the results obtained during the proliferative phase of the menstrual cycle, it was found that all study groups had an overall on average decrease in the circulating levels of cortisone during the secretory phase (figure 3.19). However, this decrease in the circulating concentration of cortisone was not deemed to be significant in all cases.

It was found using ELISA analysis that the circulating serum levels of estradiol is overall decreased in the patient study groups during the secretory phase of the menstrual cycle (figure 3.20). These levels of estradiol differed to those seen during the proliferative phase of the cycle, where the circulating levels are significantly decrease in both serum obtained

from endometriosis and UI patients compared to fertile patients ($p \leq 0.05$ and $p \leq 0.01$) (figure 3.20). Even though the concentration of estradiol is decrease in the serum isolated from ovPCOS patients, it was not significantly different to levels observed in the fertile patients.

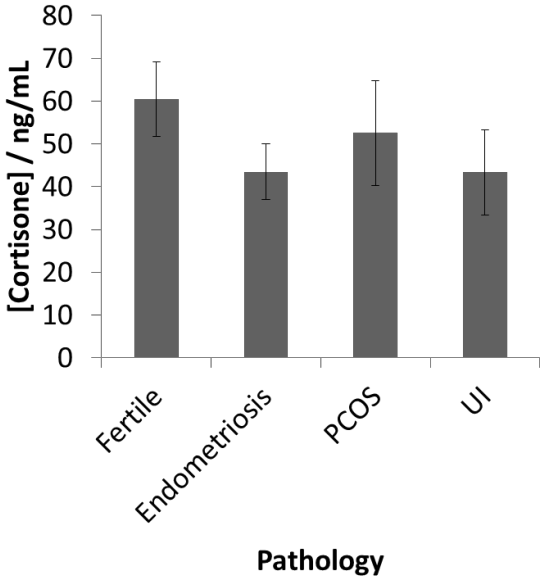


Figure 3.19 The concentration of cortisone (compound E) in the serum isolated from fertile and infertile patients during the secretory phase of the menstrual cycle. Blood samples from the fertile (n=8) and infertile patient cohorts during the secretory phase of their menstrual cycle (endometriosis n=8, PCOS n=8 and UI n=6) were centrifuged and the serum obtained was analysed for the levels of circulating cortisone using an ELISA. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ are considered significant.

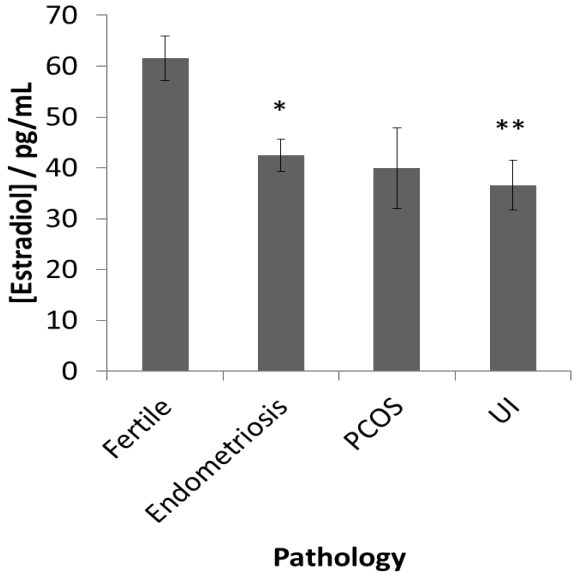


Figure 3.20 The concentration of estradiol (E_2) in the serum isolated from fertile and infertile patients during the secretory phase of the menstrual cycle. Blood samples from the (n=8) and infertile patient cohorts during the secretory phase of their menstrual cycle (endometriosis n=8, PCOS n=8 and UI n=6) were centrifuged and the serum obtained was analysed for the levels of circulating estradiol using an ELISA. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ are considered significant.

And lastly, the levels of circulating progesterone were also investigated. It was found that the circulating concentration of progesterone remains unchanged regardless of the pathology under investigation, where all results obtained for endometriosis, ovPCOS and UI patient serum using the ELISA are comparable to those observed in the fertile patient group (figure 3.21).

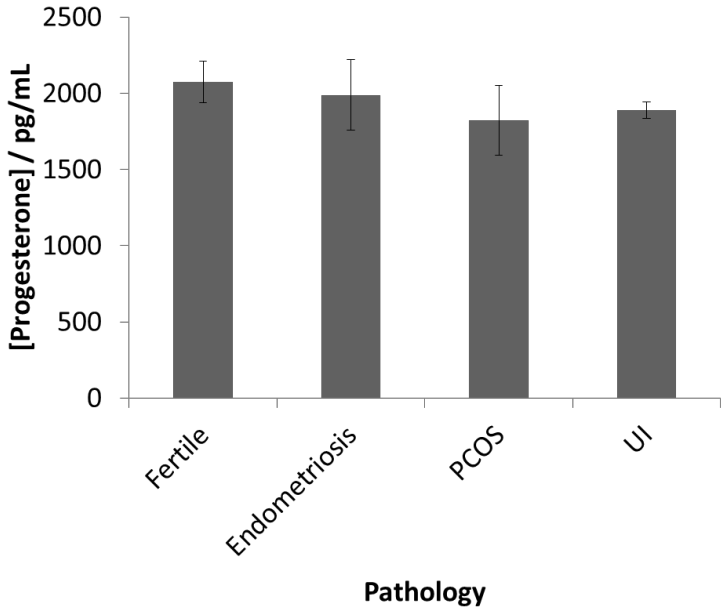


Figure 3.21 The concentration of progesterone (P_4) in the serum isolated from fertile and infertile patients during the secretory phase of the menstrual cycle. Blood samples from the fertile (n=8) and infertile patient cohorts during the secretory phase of their menstrual cycle (endometriosis n=8, PCOS n=8 and UI n=6) were centrifuged and the serum obtained was analysed for the levels of circulating progesterone using an ELISA. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

Circulating levels of androgens were only obtained for the PCOS patients during the proliferative phase of the menstrual cycle and are therefore not measured during the secretory phase.

3.2.7. Basal expression of the glucocorticoid receptor (GR) and 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes in whole biopsy samples isolated from infertile patients during the menstrual cycle

The IHC data presented in this chapter has so far has not only indicated a specific expression pattern of GR which is dependent on the endometrial location and the phase of the menstrual cycle, but it is now apparent, that this expression pattern of endometrial GR also differs between fertile and infertile patients. These results were further validated using qPCR and

immunoblot analysis to determine the basal expression levels of GR and the 11β-hydroxysteroid dehydrogenase (11β-HSD) enzyme. Whole biopsies were isolated from fertile patients and underwent tissue homogenisation followed by DNA and protein extraction.

qPCR analysis revealed that unlike results obtained for IHC, the relative gene expression of GR is not significantly altered in whole endometrial biopsies isolated from anPCOS, ovPCOS, endometriosis and UI patients during the proliferative phase of the menstrual cycle (figure 3.22A). This was also seen when investigating the relative gene expression levels of 11β-HSD1 in infertile patients compared to fertile patients, where results show that the expression of 11β-HSD1 in whole biopsies isolated from anPCOS, ovPCOS, endometriosis and UI patients is not significantly different to levels obtained in the fertile patients (figure 3.22B). However, it can be observed that there seems to be an overall decrease in the gene expression levels of 11β-HSD1 during the proliferative phase in anPCOS, ovPCOS and endometriosis patients compared to the control (figure 3.22B).

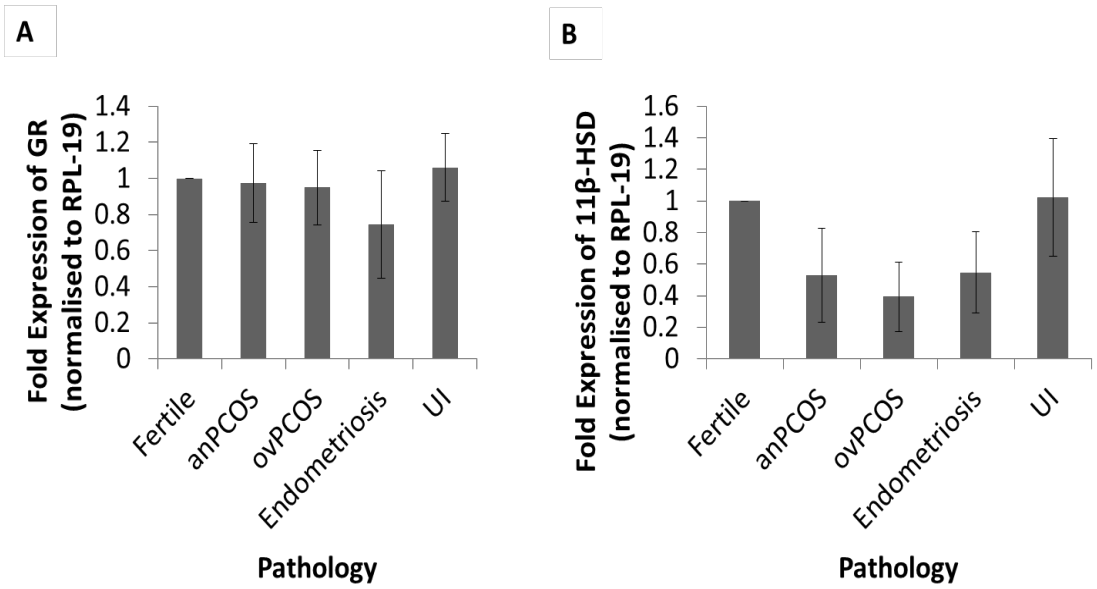


Figure 3.22. Basal mRNA expression levels of GR (A) and 11βHSD (B) in the proliferative phase of the menstrual cycle of fertile and infertile patients. Graphs show GR mRNA levels in whole tissue biopsy specimens in the proliferative phase of the menstrual cycle by qPCR. Values shown are the mean fold expression of mRNA from triplicate values per sample relative to fertile patient expression and normalised to the housekeeper gene; RPL-19. Biopsies were isolated from the fertile (n=3) and infertile patient cohorts during the proliferative phase of their menstrual cycle (endometriosis n=3, PCOS n=3 and UI n=3 Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

Following qPCR analysis, the protein extracted from the whole biopsy samples isolated from fertile and infertile patients was used for immunoblot analysis. The results obtained from the immunoblot analysis reciprocated the results obtained at the gene level using qPCR.

It was found that the basal GRα protein expression followed a similar pattern of expression to that seen at the gene level. It was found that the levels of GRα protein were not significantly altered in whole endometrial biopsies isolated from anPCOS, ovPCOS, endometriosis and UI patients during the proliferative phase of the menstrual cycle (figure 3.23A and B). However, it can be determined that on average there seems to be an overall decrease in the levels of GRα protein in all infertile patient cohorts compared to fertile patients. Interestingly, immunoblot analysis revealed that GRβ seems to have a much more varied expression in the infertile patient study groups compared to fertile patients (figure 3.23A and C). For example, protein expression of GRβ in whole endometrial biopsies isolated from anPCOS, ovPCOS and endometriosis patients is lower than the levels seen in the fertile patients during the proliferative phase of the cycle (not significant) (figure 3.23A and C). However, comparable protein expression levels were seen in UI patients when compared to the fertile patients.

Interestingly, it was found that the protein expression of 11β-HSD in whole biopsies isolated from anPCOS, ovPCOS, endometriosis and UI patients is lower than that seen in the fertile patients during the proliferative phase of the menstrual cycle. However, these were only significant for the ovPCOS and endometriosis study groups compared to fertile patients ($p \leq 0.05$) (figure 3.23A and D).

qPCR and immunoblot analysis was also carried out on whole endometrial biopsies isolated during the secretory phase of the menstrual cycle. It was found using qPCR analysis that the expression of GR in whole biopsies followed a similar pattern to the GR protein expression in the stromal region of the endometrium during the secretory phase of the cycle (figure 3.24A). It was found that there was a slight increase in the levels of GR gene expression in biopsies isolated from ovPCOS and endometriosis patients compared to fertile patients, which was not significant.

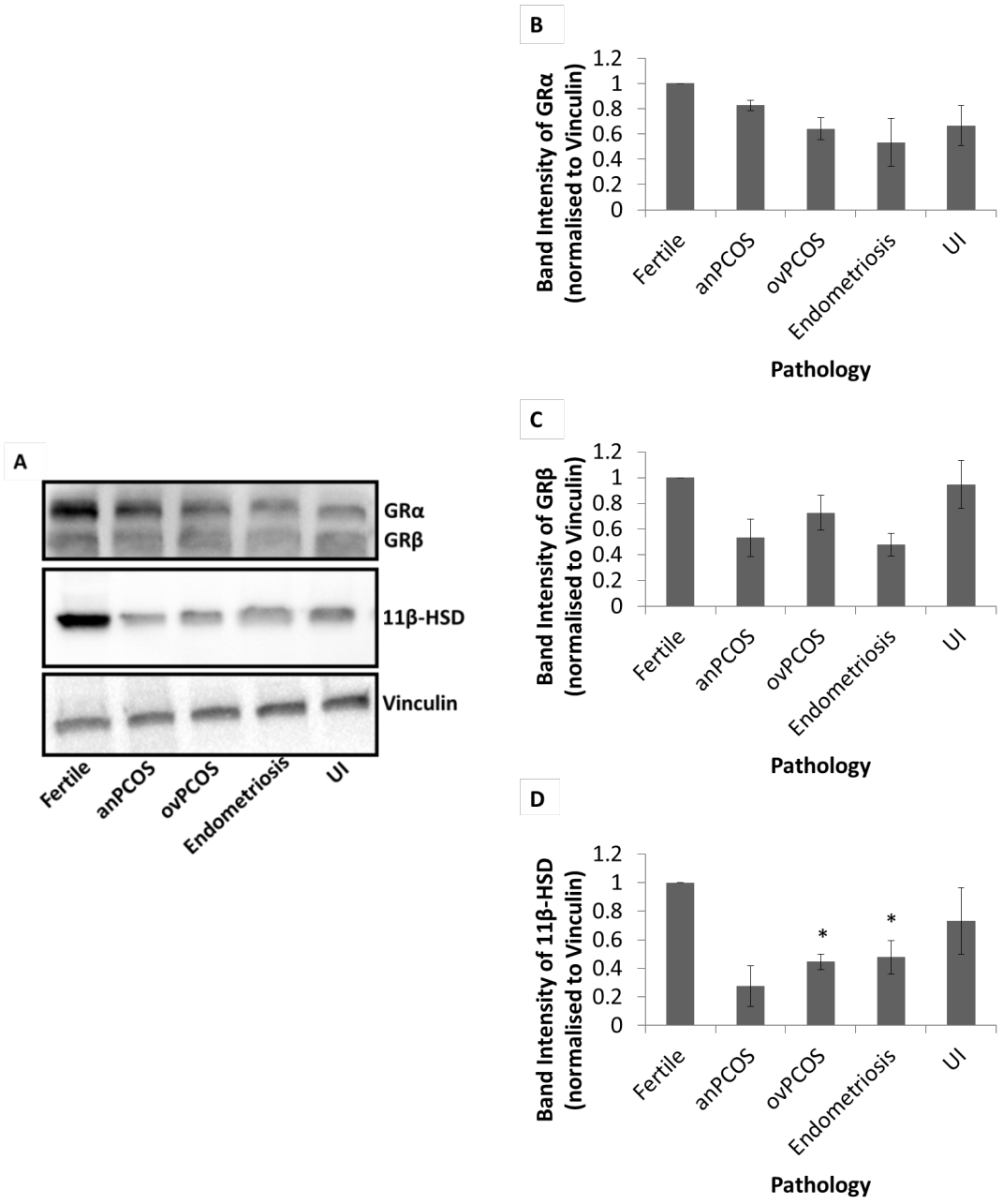


Figure 3.23. Basal protein expression levels of GRα (A and B), GRβ (A and C) and 11βHSD (A and D) in the proliferative phase of the menstrual cycle of fertile and infertile patients. Figure shows immunoblot images of GRα (95 kDa), GRβ (90 kDa), 11β-HSD (32 kDa) and the housekeeper protein; vinculin (124 kDa) in whole tissue biopsy specimens in the proliferative phase of the menstrual cycle (A). The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the house keeper protein; vinculin. Band intensity is displayed as the fold expression relative to the fertile patients and normalised to the respective vinculin samples. Biopsies were isolated from the fertile (n=3) and infertile patient cohorts during the proliferative phase of their menstrual cycle (endometriosis n=3, PCOS n=3 and UI n=3). Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

Additionally, comparable results were obtained for UI patients compared to fertile patients. Dissimilar results were obtained when the relative gene expression of 11β-HSD was investigated. It was found using qPCR analysis that the gene expression of 11β-HSD was comparable between ovPCOS and fertile patients, but there was a significant decrease in the expression of 11β-HSD in whole biopsies isolated from endometriosis and UI patients during the secretory phase of the cycle compared to the fertile patients ($p \leq 0.05$ (figure 3.24B).

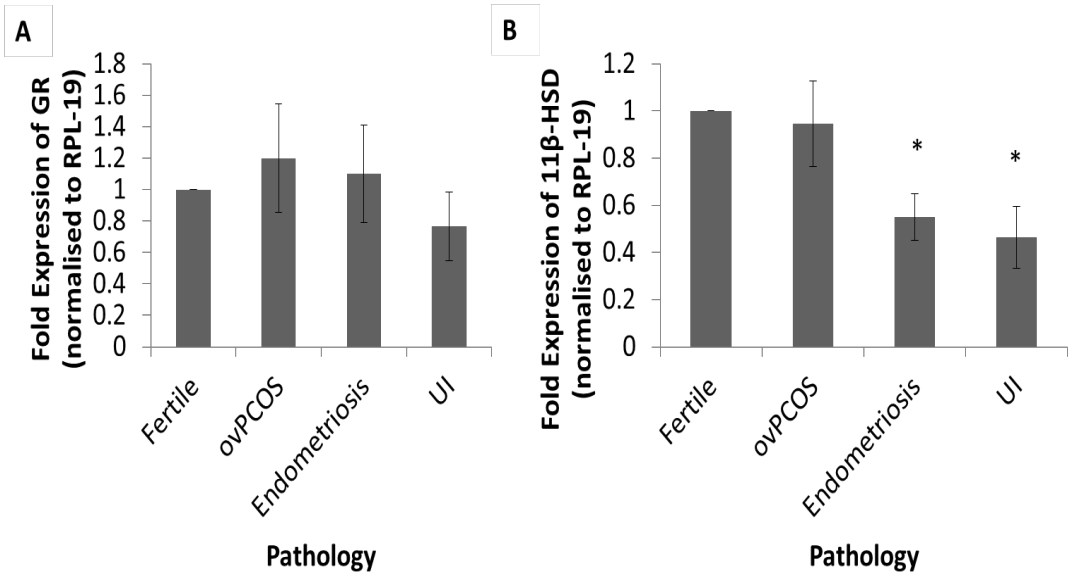


Figure 3.24. Basal mRNA expression levels of GR (A) and 11βHSD (B) in the secretory phase of the menstrual cycle of fertile and infertile patients. Graphs show GR mRNA levels in whole tissue biopsy specimens in the secretory phase of the menstrual cycle by qPCR. Values shown are the mean fold expression of mRNA from triplicate values per sample relative to the fertile patients and normalised to the housekeeper gene; RPL-19. Biopsies were isolated from the fertile (n=3) and infertile patient cohorts during the proliferative phase of their menstrual cycle (endometriosis n=3, ovPCOS n=3 and UI n=3). Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ are considered significant.

Following qPCR analysis, total protein extracted from the whole biopsy samples was analysed for GR and 11βHSD expression using immunoblotting.

The results obtained from the immunoblot analysis for GR protein expression did not reciprocate the results obtained at the gene level using qPCR. It was observed that on average there was a decrease in the expression levels of GRα compared to fertile patients during the secretory phase of the menstrual cycle and it was found that basal protein expression of GRα was lowest in the biopsies isolated from UI patients (figure 3.25A and B). In terms of statistical significance, only the biopsies isolated from ovPCOS and UI patients had a significantly lower protein expression than the fertile patients ($p \leq 0.05$ and

p≤0.01). In addition to GRα, it was also found that ovPCOS, endometriosis and UI patients have a significantly lower expression of GRβ compared to fertile patients during the secretory phase of the cycle (p≤0.05, p≤0.05 and p≤0.01) (figure 3.25A and C). Interestingly, it was found that the protein expression levels of 11β-HSD in whole biopsies isolated from ovPCOS, endometriosis and UI patients reciprocated the gene expression levels obtained from qPCR analysis. Therefore, biopsies isolated from endometriosis and UI patients exhibit a significantly lower protein expression of 11β-HSD compared to fertile patients (p≤0.05) (figure 3.25A and D). However, comparable levels of expression were observed between the ovPCOS and fertile patient study groups.

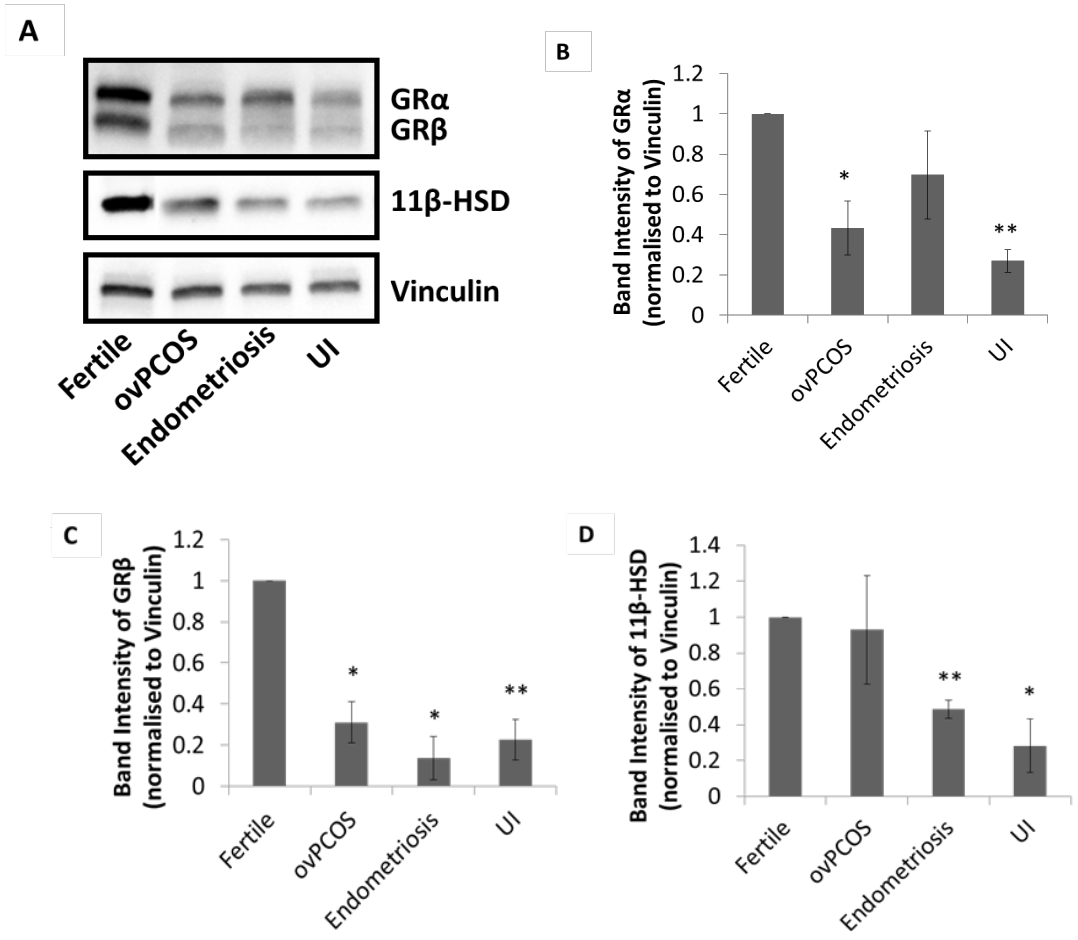


Figure 3.25. Basal protein expression levels of GRα (A and B), GRβ (A and C) and 11βHSD (A and D) in the secretory phase of the menstrual cycle of fertile and infertile patients. Figure shows immunoblot images of GRα (95kDa), GRβ (90 kDa), 11β-HSD (32 kDa) and the housekeeper protein; vinculin (124 kDa) in whole tissue biopsy specimens in the proliferative phase of the menstrual cycle (A). The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the house keeper protein; vinculin. Band intensity is displayed as the fold expression relative to fertile patients and normalised to the respective vinculin samples. Biopsies were isolated from the fertile (n=3) and infertile patient cohorts during the proliferative phase of their menstrual cycle (endometriosis n=3, ovPCOS n=3 and UI n=3). Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

3.3. Discussion

This chapter explores the basal expression levels of the glucocorticoid receptor (GR) and the 11βSD enzyme in the endometrium of fertile patients throughout the menstrual cycle phases. This was achieved by carrying out extensive immunohistochemistry (IHC) analysis, immunoblot analysis and real-time PCR (qPCR) using isolated patient biopsies. The results obtained for fertile patients were used to compare the results obtained from infertile patients, which included patients suffering from anovulatory PCOS (anPCOS), ovulatory PCOS (ovPCOS), endometriosis and unexplained infertility (UI).

Results presented in this chapter demonstrate that the expression pattern of GR in the endometrium of fertile patients differs according to the phase of the menstrual cycle and has a specific pattern of localised expression. IHC analysis revealed that the levels of GR expressed in the stromal region during the secretory phase of the menstrual cycle are significantly lower than GR expression levels within the glandular region (figure 3.2), whereas no significant differences in expression are observed during the proliferative phase of the menstrual cycle in the fertile patients (figure 3.1). Furthermore, when comparing between phases of the menstrual cycle, it is apparent that GR protein expression significantly decreases in both the glandular and stromal region of the endometrium during the secretory phase of the menstrual cycle compared to results observed during the proliferative phase (figure 3.3), which can be observed if figure 3.1 and figure 3.2 are compared visually. It is important to note that figure 3.3 indicates that GR expression within the glandular region is significantly higher than that seen within the stromal region using IHC; however this is not the case. The glandular region of the endometrium in the fertile patients has a higher 'score' after IHC analysis due to the fact that GR has a uniform distribution of staining and it was mainly differences in intensity of staining observed. Whereas within the stromal region, both reductions in intensity and the distribution of GR staining were observed, resulting in a much lower overall 'score' being obtained, this is demonstrated by the representative IHC images displayed in figure 3.1 (proliferative phase) and figure 3.2 (secretory phase).

The results obtained for endometrial GR expression in the fertile patients supported previous studies, which have also established that GR is expressed within the human endometrium throughout the menstrual cycle (Bamberger et al, 2001; McDonald et al, 2006). However, the levels and location of GR expression during the menstrual cycle observed in this study are not fully in accordance with results obtained by Bamberger and colleagues, who observed that GR is strongly expressed in the stromal region of the endometrium of patients recruited to their study, but was completely absent from the nuclei of the endometrial glands

(Bamberger et al, 2001). Whereas IHC analysis displayed in this chapter clearly showed positive GR staining within the endometrial glands in the patients recruited for this study. This is however in accordance with the work conducted by McDonald and colleagues who also found positive staining for GR in the glandular epithelium region of the endometrium, albeit it at a very low level (McDonald et al, 2006). Nevertheless, the work presented in this chapter supports the notion that there is a higher expression of GR during the proliferative phase of the menstrual cycle in the fertile patients which is clearly displayed in figure 3.3. Differences in observation between this study and the study conducted by Bamberger and colleagues, may be due to the fact that in the latter study only 9 patients were included (proliferative phase (n=5), secretory phase (n=4)), whereas this study successfully recruited and analysed biopsies retrieved from 116 patients, indicating a greater power. Of these 116 patients, 24 patients were successfully recruited as control patients (proliferative phase (n=10), secretory phase (n=14)). Additionally, the patients recruited to the Bamberger study were undergoing hysterectomies for non-endometrial diseases which included cervical carcinomas, cervical polyps and leiomyomas and can therefore not be classed as fertile patients with no disease. These pre-existing conditions may be having a significant effect on stress levels and hence the GR signalling pathway within the body, where research has shown that the presence of benign uterine tumours results in significant emotional responses in these patients, including anxiety and depression (Ghant et al, 2015). Therefore, this may have a substantial psychological impact on these patients, resulting in higher stress levels and the potential dysregulation of the GR signalling pathway. This suggests that the GR expression levels presented previously in the literature may not be 'normal' GR expression levels throughout the menstrual cycle.

In addition to investigating the basal endometrial expression of GR during the menstrual cycle, commercially available ELISA kits were used to identify whether circulating levels of cortisol and cortisone in addition to other key hormones could be correlated with endometrial GR expression levels during the menstrual cycle. ELISA results obtained in this chapter in the fertile patients, both support and contradict results obtained to date. A previous study has shown that baseline cortisol levels do not vary throughout the menstrual cycle (McCormick and Teillon, 2001), whereas results presented in figure 3.4 suggest that circulating cortisol levels do significantly vary depending on the phase of the cycle within fertile patients. This is in agreement with results obtained in 1975, which identified that circulating cortisol levels varied significantly throughout the human menstrual cycle (Genazzani et al, 1975). Results presented in chapter 3 shows that in the fertile patients, circulating cortisol levels are highest during the proliferative phase of the menstrual cycle, decrease significantly during the secretory phase and then rise again during menses (figure

3.4). This is in accordance to work conducted by Kerdelhué and colleagues who demonstrated that morning circulating levels of cortisol were higher during the proliferative phase of the cycle compared to the secretory phase (Kerdelhué et al, 2006). The changes in cortisol levels during the proliferative and secretory phase of the menstrual cycle presented here mirror the endometrial GR expression levels observed in these phases measured by IHC, where high endometrial expression of GR correlates with higher circulating levels of cortisol. Therefore, both GR and cortisol levels follow a distinct expression pattern compared to other sex steroid hormone receptors and their ligands during the menstrual cycle. The data presented here on GR and cortisol levels, supports the hypothesis that glucocorticoids (GC) regulate their own production and secretion through the feedback of the HPA axis. Hence the concentration of secreted cortisol is reduced during the secretory phase of the menstrual cycle, as a response to the peak of cortisol reported in the proliferative phase. As previously reported, higher levels of cortisol were observed during menses but no IHC data for GR expression was available for correlation analysis as biopsies were not obtained from these patients (Altemus et al, 1997).

It is important to note that the levels of circulating cortisol within the serum isolated from the recruited patients may not be truly representative. This is due to the fact that cortisol secretion is affected by many external factors, such as exercise and food, and therefore care must be taken to ensure these are accounted for (Bonen, 1976; Meulenberg et al, 1990). The results obtained in this study may be important in determining whether studies would have to control for the phase of the menstrual cycle when undergoing studies on the HPA axis or stress response. A limitation of this study is that these values are based on samples taken during one day of the menstrual cycle; therefore ideally samples would be taken throughout and across the patient's menstrual cycles. Moreover, it is known that the circulating levels of cortisol are able to alter quite rapidly, where these levels can be affected by the HPA response during sampling. For example, some patients may experience an acute stress response due to the anxiety and anticipation experienced whilst blood is being taken or levels of stress may be significantly higher than usual as the patients are currently waiting to undertake surgery. Therefore, ideally, salivary cortisol levels or urinary cortisol levels would give much more accurate results due to being less invasive. Promising results have been achieved in correlating salivary cortisol levels with the stress response throughout the menstrual cycle (Kirschbaum et al, 1999; Collins et al, 1985; Nepomnaschy et al, 2011). Therefore, care must be taken due to the effect of non-stress related factors on the concentration of circulating cortisol.

In addition to cortisol, the levels of circulating cortisone, estradiol and progesterone were also obtained during the menstrual cycle in the fertile patients. Opposing results were obtained for cortisone when compared to circulating cortisol levels, where ELISA analysis demonstrated that cortisone levels were highest during the secretory phase of the menstrual cycle, whereas cortisone levels throughout the menses and proliferative phase remained significantly lower in the fertile patients (figure 3.5). To the best of our knowledge, this is the first study at the time of writing, to report on the serum levels of cortisone throughout the menstrual cycle. Interestingly, the circulating cortisone levels followed the same pattern as those observed for the circulating levels of estradiol and progesterone during the menstrual cycle in the fertile patients (figure 3.6 and 3.7). This suggests that the phases of the menstrual cycle can be characterised by the levels of cortisol and cortisone in addition to the well-characterised sex steroid hormones. For example, the secretory phase is known to be characterised by high levels of progesterone following ovulation, falling levels of estradiol, low/absent levels of androgens and now may also be characterised by high levels of cortisone and low levels of cortisol (Maybin and Critchley, 2012). Therefore the levels of cortisol and cortisone within the secretory phase may have a significant impact on preparing the endometrium for implantation, should fertilisation occur. Regardless of results presented in figure 3.6 which need further investigation, estradiol is known to be the dominant hormone of the proliferative phase and may now also be characterised by high levels of circulating cortisol and low levels of cortisone, which may have an impact on the regeneration of the endometrium (Owen, 1975).

Contrariwise, qPCR and immunoblot results obtained using mRNA and protein respectively, isolated from whole biopsies showed that GR expression is significantly higher during the secretory phase of the menstrual cycle compared to levels obtained in the proliferative and menses phases of the cycle (figure 3.8 and 3.9) which conflicts with the IHC data presented in this chapter. This may be due to the fact that separation of the epithelial and stromal cells in the biopsy was not achieved due to the limited amount of tissue obtained, which means that the total mRNA extracted from the tissue includes all the cell types present, such as the glands, lumen and stroma. In addition to these cell types, it is also known that during the secretory phase of the menstrual cycle, there is an influx of cytokines and chemokines and leukocyte numbers increase (Maybin and Critchley, 2012). The endometrium possesses a unique immunological environment which allows for the implantation of the semi-allogeneic embryo, the influx of large populations of macrophages and uterine specific natural killer (uNK) cells infiltrating the implantation site are thought to be important in modulating trophoblast invasion and the crucial process of decidualization (Jones et al, 2004). Furthermore, recent work by Thiruchelvam and colleagues (2015) demonstrated that

endometrial macrophages express GR during the secretory and menses phases of the menstrual cycle, but not during the proliferative phase (Thiruchelvam et al, 2015). Therefore, taking this into account, the isolation of all cell types present within the primary patient biopsies may result in higher levels of GR expression being observed. Thus, these results indicate a need to investigate the levels of GR expression in isolated stromal cells to define correlation with IHC data, as it is clearly evident in figure 3.1 and figure 3.2 that GR is abundantly expressed within the endometrium and therefore all cell types within the whole biopsy sample isolated and further used for the western blot and qPCR analysis, will contribute to its abundance levels. This therefore highlights the importance of isolating specific cell types for future work.

Whole biopsy samples were also used to characterise the expression of 11β-HSD1. 11β-HSD1 followed the same expression pattern as GR, which supports the mechanism of action of the GR signalling pathway, where 11βHSD1 is able to convert inactive cortisone into the active ligand cortisol, which is in turn able to activate the GR signalling pathway and amplify GR-mediated actions (Liu et al, 2005; Chapman, Holmes and Secki, 2013). Furthermore, even though the 11ß-HSD1 expression appears to increase during the secretory phase of the menstrual cycle, there were no significant alterations in endometrial expression levels throughout the cycle. This supports previous research which elucidated that constant levels of 11β-HSD1 activity were observed across the menstrual cycle. Similar to GR, it is also known that uNK cells also express 11β-HSD1, therefore enzyme expression levels in isolated stromal cells also have to be investigated (McDonald et al, 2006).

Even though there has been extensive interest into the role of stress on reproductive function, research has been lacking into identifying the expression of endometrial GR and endometrial 11β-HSD in infertile patients. To date, this is the first study conducted to investigate whether there are any differences in the endometrial expression patterns of GR during the menstrual cycle in patients suffering from PCOS, endometriosis or UI. IHC analysis revealed that overall; the endometrial GR expression in the glandular and stromal regions during the proliferative phase is significantly lower in all infertile groups compared to fertile patients (figure 3.10 and 3.11 respectively). On the other hand, higher levels of endometrial GR expression within the stroma were observed in the infertile groups (all study groups) compared to fertile patients during the secretory phase of the menstrual cycle (figure 3.13), however no differences in GR glandular expression were observed between the study groups during the secretory phase of the menstrual cycle (figure 3.12). It must be noted that in the PCOS study group during the secretory phase of the menstrual cycle, anPCOS patients were not able to be included as all patients remain within the proliferative phase of the

menstrual cycle due to endocrine abnormalities (Meenakumari et al, 2004). Studies on the expression levels of GR on peripheral blood mononuclear cells in PCOS patients, also demonstrated a significant up-regulation of GR in this patient group which supports the IHC data presented here during the secretory phase of the cycle (Milutinović et al, 2011; Macut et al, 2010). Furthermore, research has also suggested that endometriosis patients may also experience increased expression of GR due to the presence of high levels of locally available cortisol (Monsivais et al, 2012). It is thought that high levels of GR expression in endometriotic lesions are due to the pro-inflammatory milieu of the lesions, characterised by increase macrophage infiltration and elevated cytokine levels, due to the presence of the disease (Grandi et al, 2016). The IHC data presented in this study suggest the novel finding that the endometrial GR expression pattern is altered in these infertile pathologies (PCOS, endometriosis and UI), where a higher level of GR expression is observed during the secretory phase of the menstrual cycle (figure 3.13) which contains the critical 'window of implantation' (Tapia-Pizarro et al, 2014). This suggests that the dysregulated expression of GR may be playing a role in the impaired decidualization reported for these infertile patients.

In addition to GR expression, circulating levels of cortisol and cortisone were also altered in a sub-set of patients suffering from infertile pathologies under investigation in this study. It was found that interestingly, patients suffering from PCOS and UI had significantly higher levels of circulating cortisol during the secretory phase of the menstrual cycle only (figure 3.18), whereas no significant variations in cortisol serum levels were observed during the proliferative phase of the cycle (figure 3.14). These results support previous findings which suggest that PCOS patients have increased plasma cortisol levels compared to patients with no features of PCOS (Kialka et al, 2015). Furthermore, Csemisczky and colleagues also found that infertile women who are normally menstruating and therefore may be categorised as suffering from UI, have elevated levels of cortisol compared to fertile controls (Csemiczky et al, 2000). Alterations in circulating cortisone levels were also observed, where during the proliferative phase of the cycle, there seemed to be an on average increase in the levels of cortisone observed compared to fertile patients (figure 3.15); however during the secretory phase this effect was reversed (figure 3.19). Cortisone levels in infertile patients were lower than those observed in the fertile patients, and they followed a similar pattern to circulating estradiol levels, which were are also lower during the secretory phase of the menstrual cycle compared to fertile patients. The ELISA results for the stress hormones correlated to the IHC data for endometrial GR expression in infertile patients, as seen in the fertile patients as both increased circulating levels of serum cortisol and higher levels of endometrial GR expression were observed during the secretory phase of the cycle

in infertile patients compared to fertile patients, particularly in patients suffering from PCOS or UI. These findings suggest that it is not only the dysregulation of the well-characterised sex steroid hormones, which may be having an impact on fertility status and disease pathology, but a dysregulation of the circulating levels of cortisol and cortisone particularly during the secretory phase of the menstrual cycle may also have an impact on crucial processes within the human endometrium.

Whole tissue biopsy analysis using immunoblots and qPCR experiments further supported the IHC data for infertile patients presented in this chapter during the proliferative phase of the cycle only. Immunoblot and qPCR (along with IHC) analysis revealed that endometrial GR expression during the proliferative phase of the cycle is decreased in infertile patients compared to fertile patients (figure 3.22 and 3.23). Whereas, immunoblot analysis in particular indicates that GR endometrial expression during the secretory phase of the cycle is significantly decreased in infertile patients compared to fertile patients, which contradicts the IHC data presented in this chapter. This further highlights the need to investigate the expression of GR within the primary ESCs isolated from infertile and fertile patients.

In addition to GR, whole tissue biopsies were also used to determine the expression of the 11β-HSD1 enzyme in the endometrium of infertile patients at the protein and mRNA level. Results obtained from immunoblot and qPCR analysis showed that patients suffering from PCOS seem to have a lower endometrial expression of 11β-HSD1 in the proliferative phase only of the menstrual cycle compared to fertile patients (figure 3.22, 3.23 and figure 3.24 and 3.25 respectively). Work to date has investigated the expression of 11β-HSD1 in granulosa cells (Zhu et al, 2016) and in the adipose tissue isolated from PCOS patients, where qPCR analysis revealed that 11β-HSD1 expression was significantly higher in PCOS patients in both cases (Li et al, 2013; Svendsen et al, 2009). However, due to potential differences in protein expression between tissues and cell types, the endometrial expression results obtained in this study cannot be directly compared to results obtained for adipose tissue or granulosa cell expression levels. Recent investigations have discovered that abnormal cortisol metabolism takes place in PCOS patients and there may be tissue-specific dysregulation of the 11β-HSD1 enzyme within this patient group which warrants further investigation (Gambineri et al, 2014).

Whereas endometrial expression of 11β-HSD1 in endometriosis and UI patients was observed to be lower than fertile patients during the secretory phase of the menstrual cycle. Conversely, work conducted by Monsivais and colleagues showed that cells isolated from endometriotic lesions displayed high levels of 11β-HSD1 (Monsivais et al, 2012). However, the work conducted in this study on endometriosis patients, used biopsy samples isolated

from the eutopic endometrium and not endometriotic lesions which may demonstrate why conflicting results were obtained.

Overall, the data presented here suggests that further studies need to be conducted using ESCs isolated from primary biopsy samples instead of using whole tissues biopsies which contain multiple cell types. The results also indicate that the potential dysregulated expression of GR, 11β-HSD1 and alterations in circulating levels of cortisol may have a significant role in the secretory infertile endometrium, particularly during the decidual response and implantation, which needs to be further investigated.

CHAPTER 4

The effect of glucocorticoid receptor (GR) signalling pathway activation in the human endometrium

4.1 Introduction

The endometrium is only receptive to an implanting blastocyst for a limited period of time during the menstrual cycle known as the 'window of implantation' (Giudice, 1999; Navot, 1991). This window is described as the period in the mid-secretory phase of the menstrual cycle, between days 19 and 24 and is the optimum time for successful implantation (Navot, 1991; Dominguez et al, 2003). In order for implantation to take place, a subtle dialogue between the endometrial cell types and the blastocyst is essential. The luminal epithelial cells of the endometrium are the primary barrier for the implantation process and the progesterone responses within this cellular compartment are crucial in supporting the endometrial receptive phenotype (Simon, 2003). However, it is actually the underlying stromal region of the endometrium, which mediates signals known to control the implantation process through processes such as decidualization.

Decidualization involves morphological, biochemical and vascular modifications of the endometrium and allows the endometrial cellular compartments to not only regulate the invasion of the blastocyst but to also modulate local angiogenesis, the recruitment of specific immune cells and allows for the resistance of environmental and oxidative stress (Ramathal, 2010). Defects in the decidualization process which may lead to unsuccessful implantation are thought to predispose females to pregnancy complications such as miscarriage, pre-eclampsia, foetal growth restriction and pre-term labour (Simon, 1998). Furthermore, defects in the decidualization process can even result in infertility.

It has been previously reported that both GR and 11β-HSD1 are expressed within the decidualizing endometrium specifically within the endometrial stromal cells (ESCs), whereas 11β-HSD2 expression appears to be absent during this time period (McDonald et al, 2006). Moreover, it is thought that the 11β-HSD1 expression is actually up-regulated during *in vitro* decidualization of ESCs (Kuroda et al, 2013; Takano, 2007).

Previous research conducted by Bamberger and colleagues and the results presented in chapter 3, suggest that GR is expressed within the stromal region of the endometrium and has a distinct expression pattern throughout the menstrual cycle in the fertile patients (figure 3.3). Additionally, GR also has a distinct expression compared to the other sex steroid hormone receptors during the menstrual cycle (Bamberger et al, 2001).

In the present study, GR was expressed within both the glandular and stromal region, where its levels together with circulating cortisol levels were significantly down-regulated during the secretory phase compared to the proliferative phase of the menstrual cycle (figure 3.3 and 3.4). Interestingly, IHC and ELISA analysis revealed that stromal GR expression and

circulating cortisol levels were significantly up-regulated during the secretory endometrium in infertile patients compared to fertile patients (figure 3.13 and 3.18).

Defects in decidualization during the secretory phase of the menstrual cycle have been previously reported in infertile patients, including patients suffering from PCOS and endometriosis (Gonzalez et al, 2012; Aghajanova et al, 2009). Due to significant differences in both the endometrial GR and cortisol expression patterns in fertile and infertile patients during the menstrual cycle, particularly within the secretory phase, where the crucial step of decidualization takes place, it can be hypothesised that an altered endometrial GR signalling pathway may contribute to the impaired decidualization which has been reported in infertile patients.

The role of the GR signalling pathway in the endometrium of mice has been recently investigated, which suggests that stress and GR play a pivotal role within the endometrium, which may also be transferable to the human endometrium (Whirledge et al, 2015). To date a number of epidemiological studies have suggested an association between stress exposure and the consequent activation of the HPA axis and female infertility.

For example, studies suggest that stress may be an initiator of infertility in females with an otherwise fertile endometrium. Marcus and colleagues discovered that 29% of couples under investigation conceived spontaneously over a six year period following the cessation of IVF/ICSI (Marcus et al, 2016), which may be attributable to a reduction in stress levels.

To date, little is known about the molecular effect of stress exposure and the GR signalling pathway within the human endometrium, particularly within the stromal compartment and its consequent impact on the decidualization process and fertility status. Results presented in chapter 3 identify the need to isolate ESCs from primary biopsy samples to gain a true understanding of the expression of GR, as results obtained using whole tissue biopsy samples are tainted by the presence of other infiltrating cell types. Therefore the use of ESCs will allow us to eliminate the presence of uterine macrophages which express GR and are known to be present during the decidualization process (Thiruchelvam et al, 2016). In this thesis, the critical determinant of implantation is considered to be successful decidualization and the use of isolated ESCs also allows for the replication of the progesterone dependent process of decidualization *in vitro* using cAMP (Ramathal, 2010). The *in vitro* decidual response can be measured quantitatively via morphological changes of the ESCs and the expression of key decidual markers.

This chapter focuses on expanding the work conducted in chapter 3 by aiming to determine the endometrial stromal expression of GR and the effect of the GR signalling pathway in the presence and absence of the stress hormones, during the crucial process of decidual

transformation, in otherwise fertile endometrium. This will be conducted using ESCs isolated from fertile patients instead of using whole tissue biopsies as was carried out in chapter 3. Furthermore, this chapter investigates whether the HESC cell line can be used as a suitable *in vitro* model of the fertile endometrium to assess the GR signalling pathway during decidual transformation. This was necessary due to the limited amount of primary cells available for chromatin isolation, which requires a starting material of 1000-15million cells. Finally, if HESC proves to be a suitable model of endometrial response to GCs during decidual transformation, targets of the GR signalling pathway will be elucidated and identified using this cell line.

4.2 Results

4.2.1 Basal expression of the glucocorticoid receptor (GR) and 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes in endometrial stromal cells

The expression of GR and 11β-HSD1 has previously been described within the human endometrium (Bamberger et al, 2001; McDonald et al, 2006; Kuroda et al, 2013). Initial experiments were performed to determine the basal protein and gene expression levels of GR and the 11β-HSD enzymes in the HESC cell line (established immortalised endometrial stromal cell line) and primary endometrial stromal cells (ESCs) isolated from fertile patients.

Initial results showed that both the HESC cell line and ESCs isolated from fertile patients expressed GRα, GRβ, 11β-HSD1 and 11β-HSD2 at the protein level (figure 4.1A and B) and GR, 11β-HSD1 and 11β-HSD2 at the gene level (figure 4.2). Differences in levels of expression of GR and 11β-HSD can be seen between the HESC cell line and the primary ESCs.

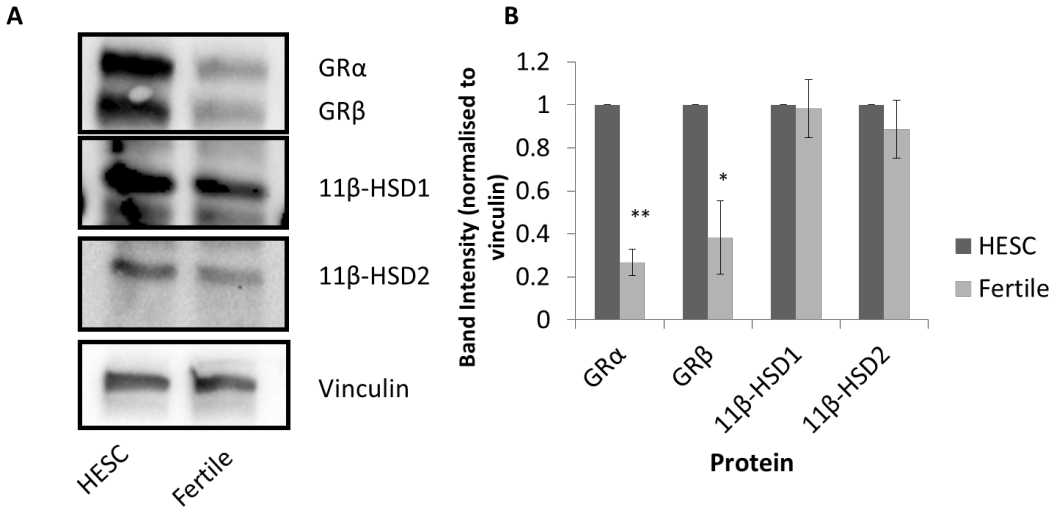


Figure 4.1A and 4.1B. Basal protein expression levels of GR and 11βHSD in HESCs and ESCs isolated from fertile patients. A) Image shows the representative images of western blot analysis of GRα (95kDa), GRβ (90kDa), 11βHSD1 (32 kDa) and 11βHSD2 (40kDa) protein levels in ESCs isolated from fertile patients (indicated by 'fertile') (n=5) compared to the HESC cell line (n=3). B) The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the house keeper protein; vinculin. Band intensity is displayed as the fold expression relative to the HESC cell line and normalised to the respective vinculin samples. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

It was found that the HESC cell line had a significantly higher basal protein expression of both GRα and GRβ compared to ESCs isolated from fertile patients ($p \leq 0.01$ and 0.05 respectively). However, there was no significant difference seen in GR expression at the mRNA level. It is important to note that the gene specific primers designed to recognise GR, recognises both α and β isoforms.

In comparison, there were no significant differences in the basal expression levels of both 11β-HSD1 and 11β-HSD2 between the HESC cell line and ESCs isolated from fertile patients, at both the protein and gene level. However the gene levels of the 11β-HSD enzymes follow a similar pattern to that seen at the protein level.

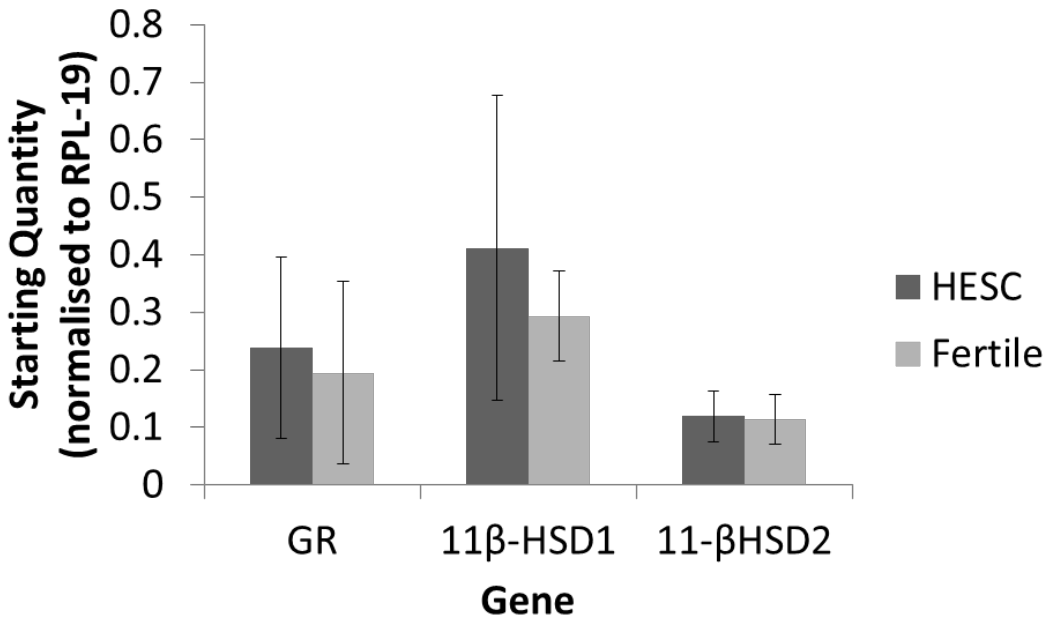


Figure 4.2. Basal mRNA expression levels of GR and 11βHSD in HESCs and ESCs isolated from fertile patients. Graph shows the GR and 11B-HSD mRNA levels in the HESC cell line (n=3) and ESCs isolated from fertile patients (n=5) by qPCR analysis. Values shown are the mean starting quantity from triplicate values per sample, normalised to the housekeeper gene; RPL-19. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

This data therefore suggests that ESCs isolated from fertile patients express both isoforms of GR, particularly GRα which is able to be activated by ligand binding and can alter the transcription of key target genes. ESCs also express both 11β-HSD and 11β-HSD2, allowing the ESCs to participate in the cortisol-cortisone shuttle (REF). Results shown in figure 4.1 and 4.2 also indicate that the HESC cell line is a good model cell line due to the similar expression patterns seen at both the protein and gene level.

Despite this, the effect of the biologically active ligand cortisol and its inactive counterpart, cortisone on ESCs has to be further confirmed.

4.2.2 The effect of cortisol during decidual transformation of endometrial stromal cells

Due to the fact that ESCs express both GRα and GRβ and the enzymes required to control the local bioavailability of cortisol it was decided to investigate the role of corticosteroid and

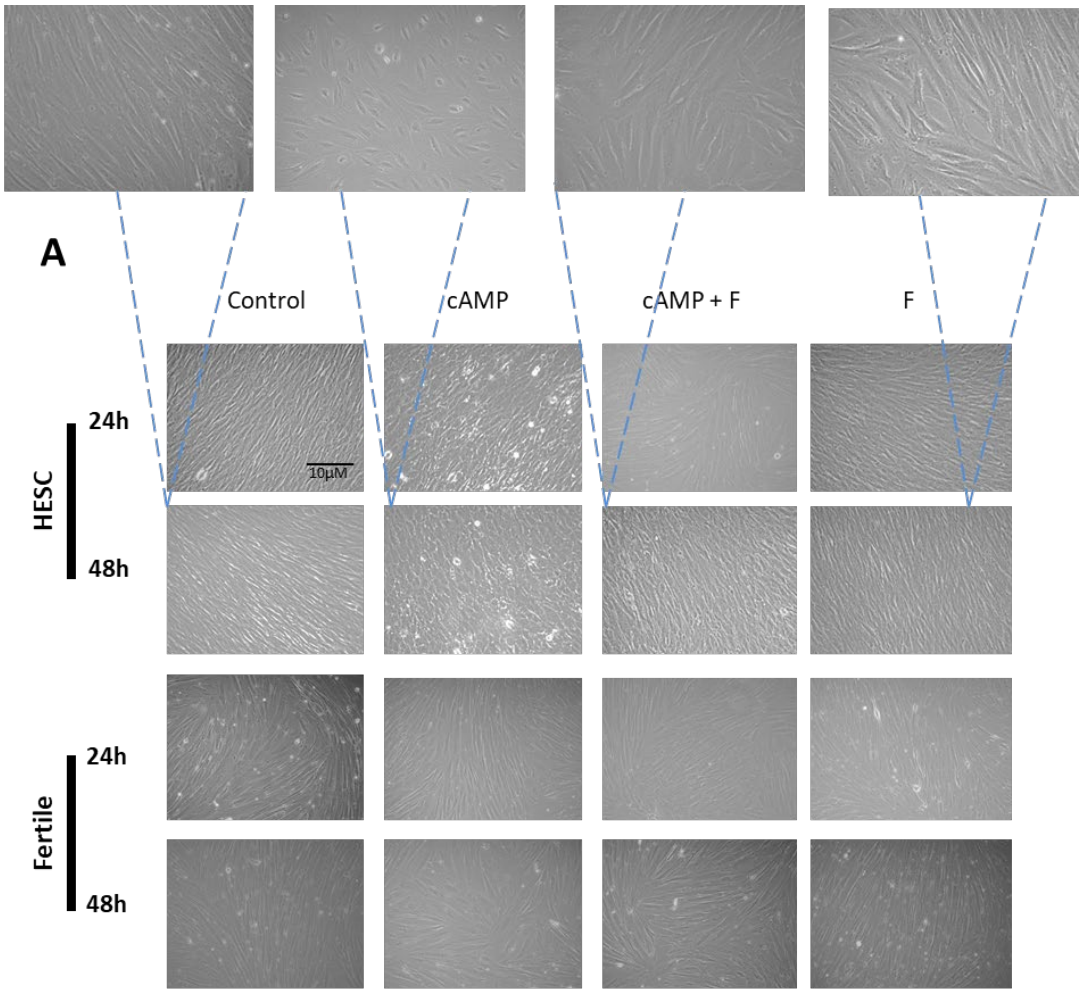
GR signalling during the decidualization. Therefore to explore the role of cortisol and the GR signalling pathway on decidual transformation of the stromal cells, the HESC cell line and ESCs isolated from fertile patients were cultured and treated with the *in vitro* decidualization stimulus in combination with the stress hormone, cortisol for 48 h.

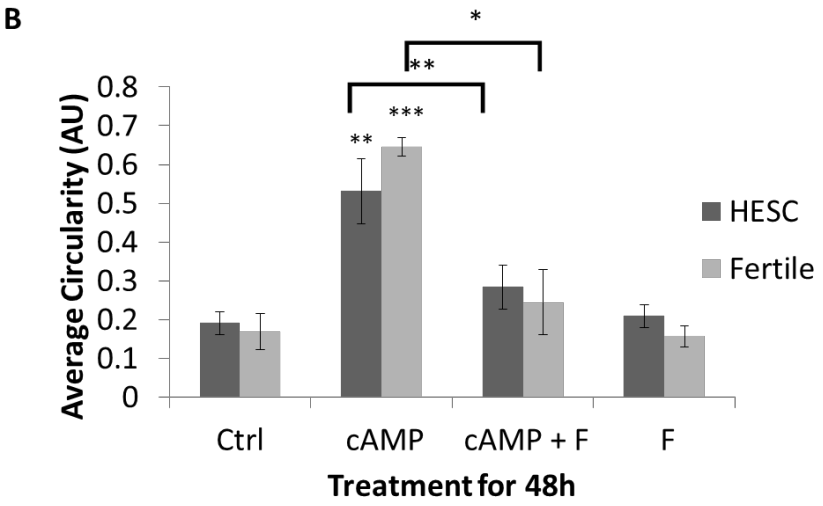
Decidualization was determined based on the morphological appearance and changes in the stromal cells and by the expression of key decidual markers. This comprises of a change in fibroblast shape cells to more rounded epithelial like cells and an induction of expression of dPRL and IGFBP-1.

4.2.2.1 The effect of cortisol on cell morphology and the expression of key decidual markers during decidual transformation

Cell monolayers were treated for 48 h with cAMP, cAMP + cortisol (F) or F and compared to control cells. It was found that treatment with the *in vitro* decidualization stimulus; cAMP, caused a significant change in cell morphology, where cells adopted a more rounded epithelial like phenotype compared to their normal fibroblastic phenotype compared to the control (figure 4.3 A and B).

This visual change in morphology seen in figure 4.3A after cAMP treatment indicates that the stromal cells have undergone successful decidual transformation in vitro. This was further confirmed quantitatively using Image J analysis, where it was found that the both the 'HESC' cells and ESCs isolated from fertile patients treated for 48 h with cAMP had a significant increase in circularity from approximately 0.18 and 0.15 to 0.5 and 0.65 respectively ($p \leq 0.01$; $p \leq 0.001$).


A
Control
cAMP
cAMP + F
F
HESC
24h
48h
10μM
Fertile
24h
48h


B
*
**
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Average Circularity (AU)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
HESC
Fertile
Ctrl
cAMP
cAMP + F
F
Treatment for 48h

Figure 4.3A and 4.3B. The effect of cortisol (F) on the morphology and circularity values of the HESC cell line and ESCs isolated from fertile patients during decidual transformation. A) Representative images of the cell monolayers after 48 h of treatments using inverted light microscopy (Zeiss) at an x10 objective. Scale bar indicates 10μM. Insets of representative images taken at a x20 objective are also shown for some treatment groups. These images were then used for Image J analysis to gain a quantitative measure of cell morphology. B) Thirty cells were chosen at random from each image taken of the HESC cell line (n=3) and ESCs isolated from fertile patients (n=5), therefore 90 cells were analysed from each treatment group for the HESC cell line and 150 cells were analysed from the ESCs isolated from fertile patients from each treatment group. Values are averaged from 90 cells (HESCs) and 120 cells (ESCs isolated from fertile patients) and bars indicate ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ are considered significant.

It is important to note that a normal ESC (whether derived from a cell line or primary tissue) has an average circularity value of approximately 0.2, whereas ESCs which have undergone successful decidualization have an increased circularity value ranging from 0.5 – 1.0, where a circularity value of 1.0 indicates a perfect circle. These results suggest that *in vitro* decidualization success rates are comparable between the HESC cell line and ESCs isolated from fertile patients. Interestingly, it was also demonstrated that co-treatment with cAMP and F caused a significant reduction in average cell circularity values in both the HESC cell line and ESCs isolated from fertile patients compared to those treated with cAMP alone ($p \leq 0.01$; $p \leq 0.05$). Co-treatment with cAMP and F resulted in comparable average circularity values with the untreated cells. These alterations in cell morphology can be clearly seen visually in figure 4.3A. Treatment with F alone caused no significant difference in the average circularity values compared to the control in both the HESC cell line and ESCs isolated from fertile patients (figure 4.3).

To further support the morphological changes seen in these cells – the concentration of key secreted decidual markers were measured using commercially available ELISA kits.

It was found that treatment of both the HESC cell line and ESCs isolated from fertile patients with cAMP alone and co-treatment with cAMP and F caused a significant increase in the concentration of secreted prolactin compared to the control ($p \leq 0.05$ and $p \leq 0.05$; $p \leq 0.05$ and $p \leq 0.05$) (figure 4.4A). However, even though stromal cells co-treated with cAMP and F caused an increase in the secretion of prolactin, there was a notable significant decrease in these levels compared to cells treated with cAMP alone in both the HESC cell line and ESCs isolated from fertile patients ($p \leq 0.05$ and $p \leq 0.05$). It was found that HESCs and ESCs isolated from fertile patients when treated with cAMP, secrete approximately 13 fold and 19 fold more prolactin respectively compared to the control. Co-treatment with cAMP and F resulted in only a 5.5 and 8 fold increases in the levels of secreted prolactin compared to the control in HESCs and ESCs isolated from fertile patients respectively. Therefore HESCs and ESCs isolated from fertile patients treated with cAMP alone secrete

approximately 2.5 fold more prolactin than cells co-treated with cAMP and F ($p \leq 0.05$), with average secreted concentrations of prolactin of HESCs at 579 pg/mL and 236 pg/mL and ESCs isolated from fertile patients at 790 pg/mL and 312 pg/mL respectively.

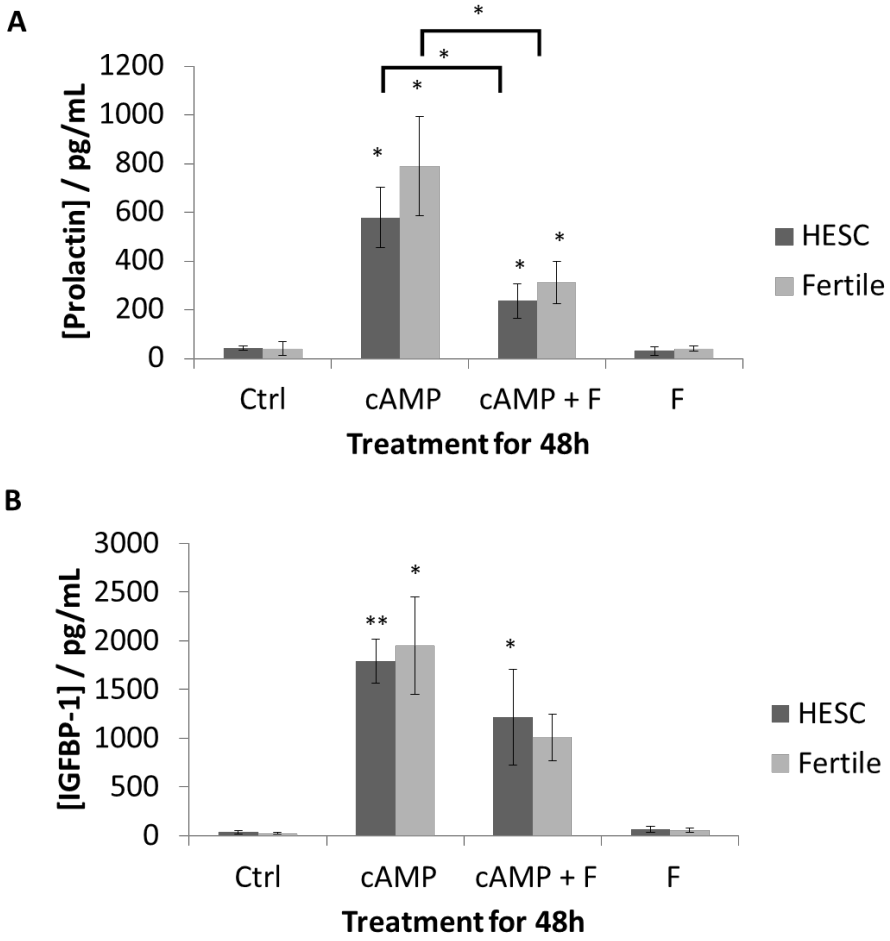

Figure 4.4. The effect of cortisol (F) on the levels of secreted decidual markers – prolactin (A) and IGFBP-1 (B) from the HESC cell line ESCs isolated from fertile patients during decidual transformation. Media was extracted after the HESC cell line (n=3) and ESCs isolated from fertile patients (n=5) underwent 48 h of treatment with either cAMP, cAMP and F or F alone and compared to the control. ELISA experiments were carried out on the conditioned media to establish the concentration of key proteins at the secreted level. Treatment with cAMP caused a significant increase in the secreted levels of both decidual markers compared to the control. This induction of secretion is reduced when stromal cells are co-treated with cAMP and F. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ are considered significant.

Similar results were obtained for secreted levels of another key decidual marker; IGFBP-1. It was found that treatment of the HESC cell line with cAMP alone caused a significant increase in the concentration of secreted IGFBP-1 compared to the control ($p \leq 0.01$), however co-treatment with cAMP and F resulted in an increase in the secreted levels of

IGFBP-1 which was not statistically significant (figure 4.4B). ESCs isolated from fertile patients treated with both cAMP alone and co-treated with cAMP and F resulted in a significant increase in the concentration of IGFBP-1 compared to the control ($p \leq 0.05$ and $p \leq 0.05$), comparable to the secreted levels of prolactin. However, in comparison to prolactin, even though stromal cells co-treated with cAMP and F caused an increase in the secretion of prolactin (significant only for ESCs isolated from fertile patients) compared to the control (untreated primary stromal cells isolated from fertile patients), which was lower than the levels seen in cells treated with cAMP alone, there was no statistically significant differences in the concentration of secreted IGFBP-1 from cells treated with cAMP alone or co-treated with cAMP and F. It was found that HESCs and ESCs isolated from fertile patients when treated with cAMP, secrete approximately 51 fold and 97 fold more IGFBP-1 respectively compared to the control. Co-treatment with cAMP and F resulted in only a 34 fold and 50 fold increase in the levels of secreted IGFBP-1 compared to the control in HESCs and ESCs isolated from fertile patients respectively. Therefore HESCs and ESCs isolated from fertile patients treated with cAMP alone secrete approximately 1.5 fold more IGFBP-1 than cells co-treated with cAMP and F, with average secreted concentrations of prolactin of HESCs at 1789 pg/mL and 1217 pg/mL and ESCs isolated from fertile patients at 1948 pg/mL and 1006 pg/mL respectively.

These ELISA results were further confirmed using qPCR to determine the expression levels of dPRL and IGFBP-1 at the mRNA level using gene specific primers. Analysis of the results indicated that both dPRL and IGFBP-1 expression was induced significantly in HESCs treated with both cAMP alone and co-treated with cAMP and F compared to the control ($p \leq 0.05$ and $p \leq 0.05$; $p \leq 0.05$ and $p \leq 0.01$), therefore supporting the results obtained using the ELISA experiments (figure 4.5A and B). This was also seen in ESCs isolated from fertility patients, but, treatment with cAMP alone only caused a significant increase in the expression levels of IGFBP-1 ($p \leq 0.05$) and treatment with cAMP and F caused a significant increase in both the expression levels of dPRL and IGFBP-1 ($p \leq 0.05$ and $p \leq 0.001$). However, unlike the ELISA results – there was no significant difference in expression of either dPRL or IGFBP-1 at the mRNA level in both study groups treated with cAMP and F compared to those treated with cAMP alone (figure 4.5). Indicating that cAMP and F treatment only statistically alters the secreted levels of the decidual proteins during decidual transformation of the ESCs.

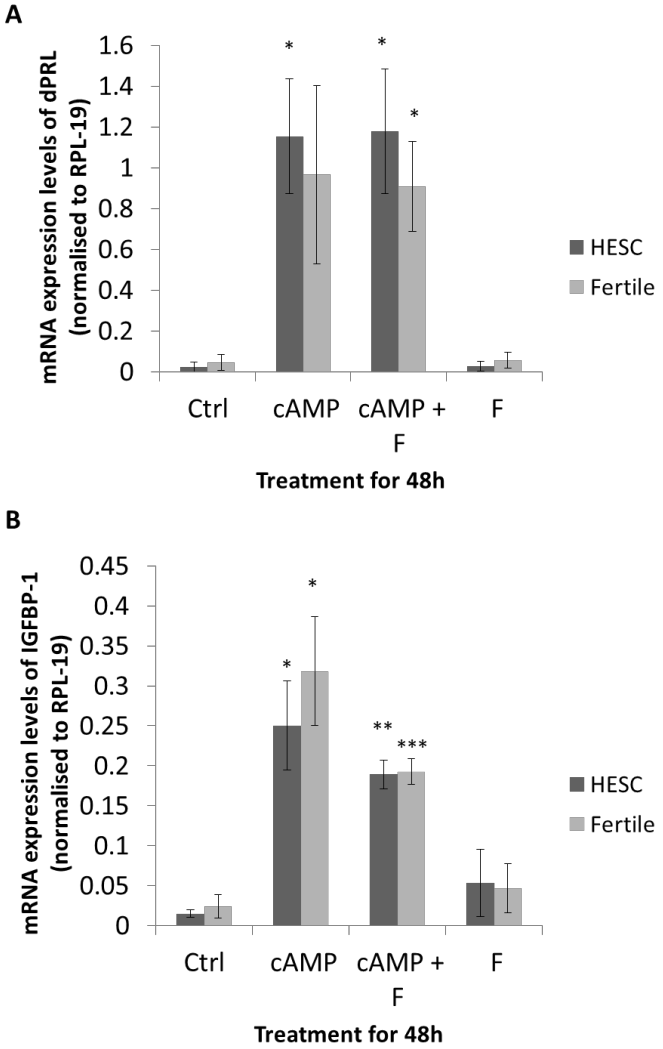

Figure 4.5 The effect of cortisol (F) on mRNA expression levels of key decidual markers – decidual prolactin (dPRL) (A) and IGFBP-1 (B) from the HESC cell line and ESCs isolated from fertile patients during decidual transformation. After 48 h of treatment the HESC cells (n=3) and ESCs isolated from fertile patients (n=5) were lysed, RNA extracted and converted to cDNA and used for qPCR analysis. Cells underwent 48h of treatment with either cAMP, cAMP and F or F alone and compared to the control. qPCR analysis was carried out to establish the mRNA expression levels of dPRL (A) and IGFBP-1 (B). Treatment with cAMP alone and cAMP and F, caused an increase in the expression levels of both decidual markers compared to the control. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

4.2.2.2 The effect of cortisol on the expression of GR and 11β-HSD during decidual transformation

Due to the pronounced effects that cAMP and F treatment was having on the ESC morphology and the expression of key decidual markers, it was decided that the role of GR and the 11β-HSD enzymes during decidual transformation in the absence and presence of cortisol should be investigated. As previously described, the HESC cell line and ESCs

isolated from fertile patients were cultured and treated with either cAMP, cAMP and F or F alone for 48 h and compared to the control.

Previous results in this chapter suggest that undifferentiated HESCs and ESCs isolated from fertile patients abundantly express GRα and GRβ at the basal level. Furthermore, *in vitro* decidualization did not result in significant difference in the expression of GRα and GRβ at the protein level in the HESC cell line (figure 4.6A, B and C). However, co-treatment with cAMP and F, and F alone resulted in a decrease in the expression of both GR isoforms in the HESC cell line compared to the control (significant only for cAMP and F treatment – $p \leq 0.05$). Co-treatment with cAMP and F resulted in a decrease of expression of both GRα and GRβ in the HESC cell line, compared to cells treated with cAMP only ($p \leq 0.01$ and $p \leq 0.01$). Primary ESCs isolated from fertile patients treated with cAMP resulted in an increase in the expression of both GRα and GRβ isoforms in the primary ESCs at the protein level compared to the control, but was deemed to be not statistically significant. And similarly, treatment with either cAMP and F, or F alone resulted in a significant decrease in GRα ($p \leq 0.01$ and $p \leq 0.05$) compared to the control, but interestingly not GRβ (figure 4.6 A, B and C). Co-treatment with cAMP and F resulted in a significant decrease in expression of only the GRα isoforms in the primary ESCs compared to cells treated with cAMP only ($p \leq 0.01$).

In addition to GR, previous results in this chapter suggested that undifferentiated HESCs and ESCs isolated from fertile patients abundantly express 11β-HSD1 at the basal level and also 11β-HSD2 but to a lesser extent. It was found that *in vitro* decidualization of the HESC cell line resulted in a significant induction of 11β-HSD1 expression at the protein level compared to the control ($p \leq 0.05$) (figure 4.6 A and D). This effect was dampened by the presence of F, as co-treatment with cAMP and F resulted in a significant decrease in the expression of 11β-HSD1 in the HESC cell line compared to cells treated with cAMP only ($p \leq 0.05$) (figure 4.6 A and D).

Interestingly, treatment with F alone resulted in a significant decrease in the expression of 11β-HSD1 in the HESC cell line, compared to the control ($p \leq 0.05$). Similar results were obtained with the primary ESCs isolated from fertility patients; however, instead of an induction of expression of 11β-HSD1 with cAMP treatment, the primary ESCs had a slight reduction in their expression levels compared to the control. The same expression patterns were seen with F treatment alone as were seen with the HESC cell line, which resulted in a significant reduction in the expression of 11β-HSD1 compared to the control ($p \leq 0.05$).

On the other hand, western blot analysis showed that low basal levels of 11β-HSD2 are not augmented regardless of treatment, in either the HESC cell line or in the ESCs isolated from

fertile patients. However, treatment with cAMP alone and co-treatment with cAMP and F actually resulted in a significant decrease in the expression levels of 11β-HSD2 in the HESC cell line compared to the control ($p \leq 0.05$ and $p \leq 0.05$). This effect was not reciprocated in the primary ESCs, where no significant differences in expression were seen throughout the treatment groups.

To further support the results obtained at the protein level using western blot analysis, qPCR was undertaken to see if the transcript levels reflected the translated protein levels.

qPCR analysis reciprocated the results seen at the protein level (figure 4.7). Treatment with cAMP alone resulted in a significant decrease in the expression of GR compared to the control ($p \leq 0.01$). Co-treatment with cAMP and F, and F alone also resulted in a decrease in the expression of GR, where only F treatment alone was deemed to be statistically significant compared to the control ($p \leq 0.01$) (figure 4.7A). The same pattern of expression of GR was obtained in ESCs isolated from fertile patients, where GR transcript levels were reduced by the treatment with cAMP, co-treatment with cAMP and F and also F alone, but these were not significantly different from the control group.

11β-HSD1 mRNA levels were undoubtedly increased by cAMP treatment in both the HESC cell line and in the primary ESCs compared to the control ($p \leq 0.001$ and $p \leq 0.05$) (figure 4.7B). However, the addition of F along with cAMP had little or no effect on the expression levels in cAMP treated cells as there was no difference between 11β-HSD1 expression levels between the cells treated with cAMP and those treated with cAMP and F for 48 h.

Expression of 11β-HSD2 mRNA was low in the HESC cell line and in ESCs isolated from fertile patients during decidual transformation (figure 4.7C). Similar to the protein data, treatment with cAMP, cAMP and F and also F alone resulted in a significant decrease in the expression of 11β-HSD2 at the mRNA level compared to the control in the HESC cell line ($p \leq 0.01$). This significant difference in expression was not observed in the primary cells.

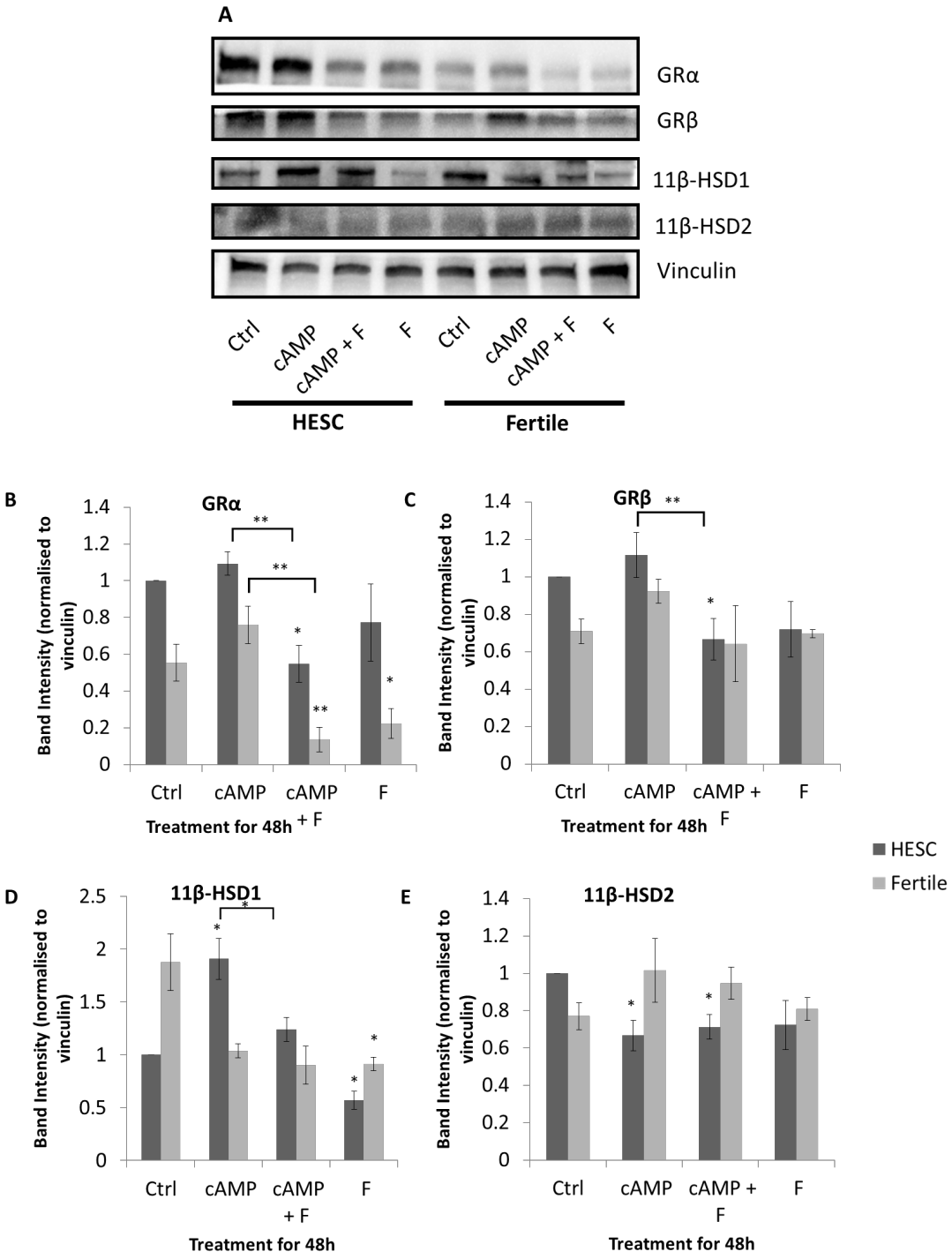

Figure 4.6 The effect of cortisol (F) on key proteins involved in the GR signalling pathway during decidual transformation in the HESC cell line and ESCs isolated from fertile patients. A) Representative images show the western blot analysis of GRα (95kDa), GRβ (90kDa), 11βHSD1 (32 kDa) and 11βHSD2 (40 kDa) protein levels in ESCs isolated from fertile patients (n=5) vs the HESC cell line (n=3) after 48h treatment with either cAMP, cAMP & F or F alone and compared to untreated control. B-E) The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the housekeeper protein; vinculin (124 kDa). Band intensity is displayed as the fold expression relative to the control HESC cells and normalised to the respective vinculin samples. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

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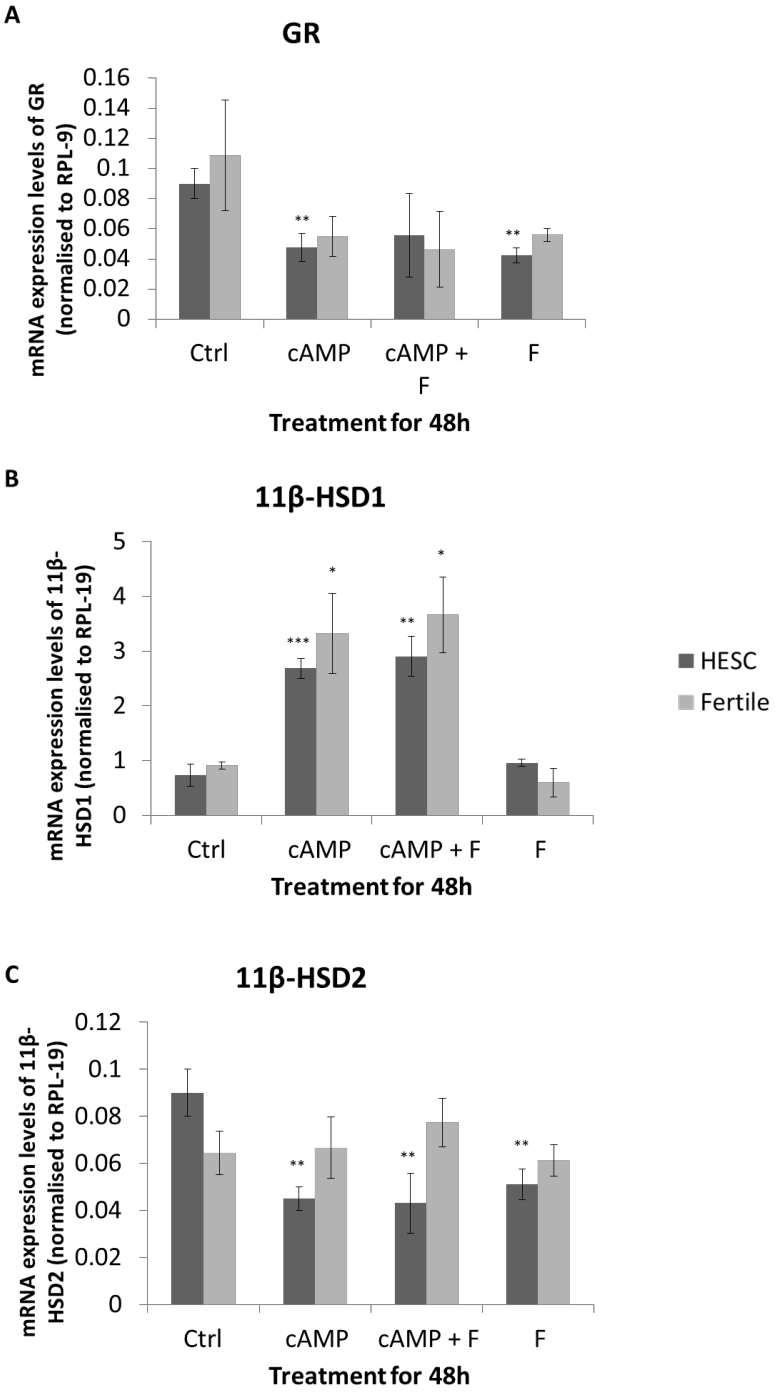


Figure 4.7 The effect of cortisol (F) on mRNA expression levels of GR (A), 11β-HSD1 (B) and 11β-HSD2 (C) in the HESC cell line and ESCs isolated from fertile patients during decidual transformation. After 48h of treatment, the HESC cells (n=3) and ESCs isolated from fertile patients (n=5) were lysed, RNA extracted and converted to cDNA and used for qPCR analysis. Cells underwent 48 h of treatment with either cAMP, cAMP and F or F alone and compared to the control. qPCR analysis using gene specific primers was carried out to establish the expression levels at the mRNA level of GR (A) and 11β-HSD1 (B) and 11β-HSD2 (C). Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ are considered significant.

Due to the known homology between GR and the mineralocorticoid receptor (MR), and also due to previous results reported by McDonald et al (2006), indicating that MR is up-regulated during the secretory phase of the menstrual cycle. Preliminary results using qPCR investigated whether MR expression was altered during decidual transformation, where it was found that MR seems to follow the reverse pattern of GR as its expression seems to increase during decidual transformation in the HESC cell line (figure 4.8). This suggests a switch from GR to MR activation in ESCs during decidualization.

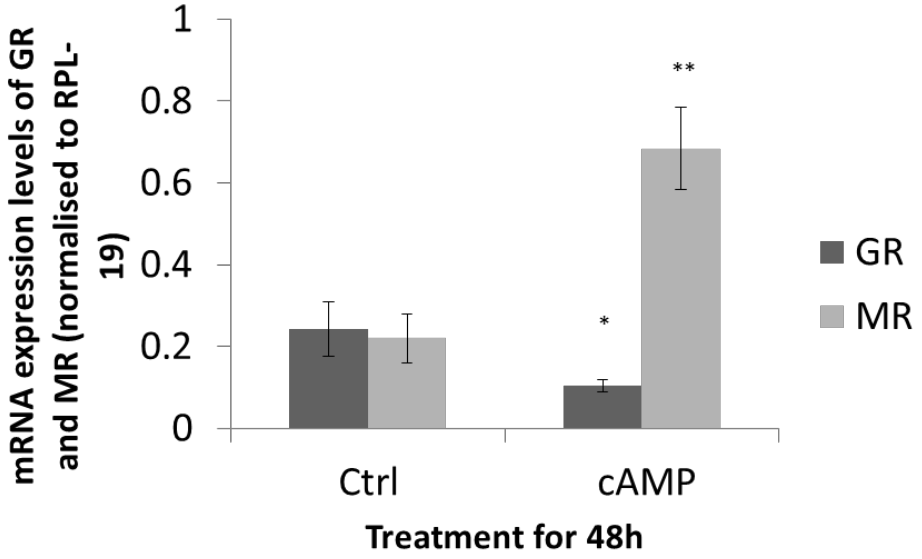


Figure 4.8 Preliminary result showing the effect of in vitro decidualization of the HESC cell line on mRNA levels of GR and MR. After 48 h of treatment, the HESC cells (n=3) were lysed, RNA extracted and converted to cDNA and used for qPCR analysis. Cells underwent 48 h of treatment with either cAMP or were left untreated. qPCR analysis using gene specific primers was carried out to establish the mRNA level of GR and MR. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

4.2.2.3 The effect of cortisol (F) on the expression of potential GR targets during decidual transformation

Due to the apparent alteration in the GR signalling pathway within the ESCs during decidual transformation, it was decided to investigate the effect of cortisol on the expression of a selection of genes which are essential within the human endometrium particularly during decidualization. These include the Wilms tumour suppressor gene (*WT1*), a known gatekeeper of decidualization (Gonzalez et al, 2012) and the immunophilins; FK506 binding proteins 4 and 5 (FKBP4 and FKBP5) crucial co-chaperone proteins of the steroid hormone receptors, which were reported to be genes critical to implantation and decidualization using knock out mice models (Gellersens and Brosens, 2014) (figure 4.9).

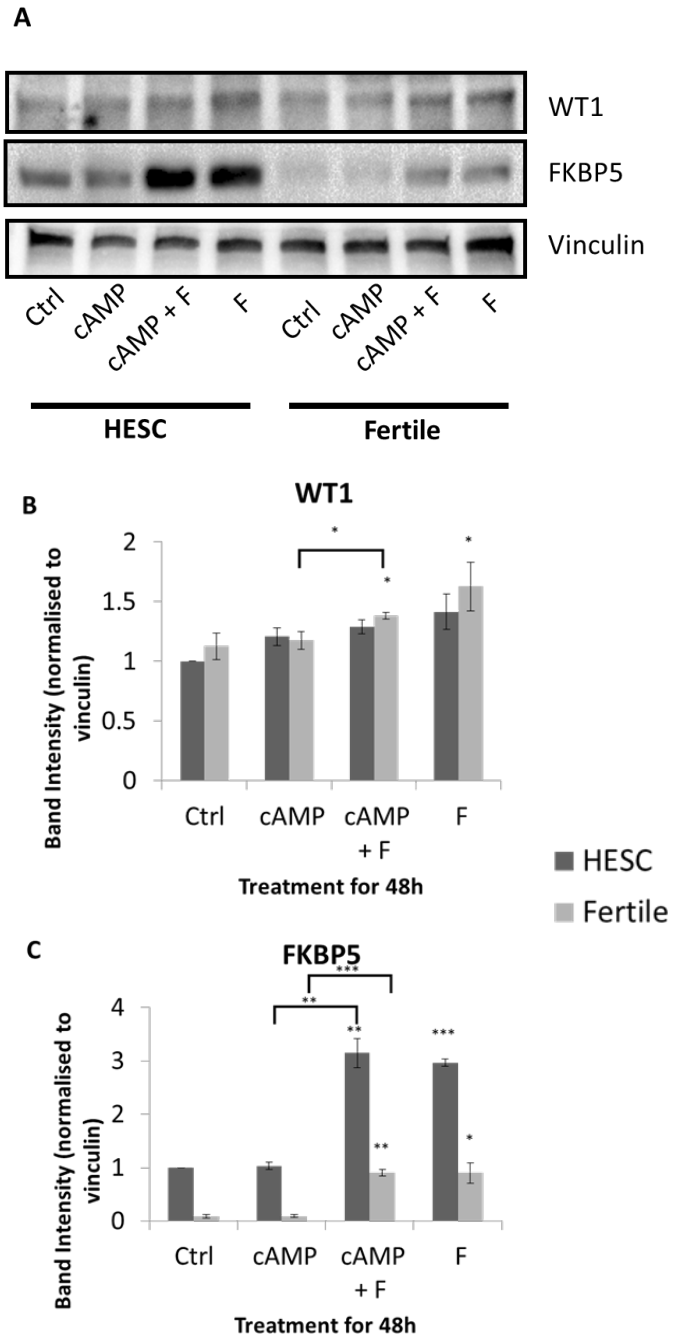


Figure 4.9 The effect of cortisol (F) on WT1 and FKBP5 protein expression during decidual transformation of the HESC cell line and ESCs isolated from fertile patients. A) Representative images show the western blot analysis of WT1 (52 kDa), FKBP5 (51 kDa) and vinculin (124 kDa) protein levels in ESCs isolated from fertile patients (n=5) vs the HESC cell line (n=3) after 48h treatment with either cAMP, cAMP and F or F alone and compared to the untreated control. B-C) The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the housekeeper protein; vinculin. Band intensity is displayed as the fold expression relative to the control HESC cells and normalised to the respective vinculin samples Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

In vitro decidualization resulted in no significant difference in expression of either WT1 or FKBP5 at the protein level in either the HESC cell line or ESCs isolated from fertile patients (figure 4.9). However co-treatment with cAMP and F resulted in an increase in WT1 protein expression in the HESC cell line compared to both the control and cAMP treatment (not significant in both cases). This effect was also seen in the primary ESCs, where co-treatment with cAMP and F resulted in a significant increase in the expression of WT1 compared to the control ($p \leq 0.05$) and compared to cells treated with only the decidualization stimulus; cAMP ($p \leq 0.05$). Interestingly, treatment with F alone resulted in a significant increase in WT1 expression in the primary ESCs compared to the control ($p \leq 0.05$) (figure 4.9 A and B).

Furthermore, it was found that co-treatment with cAMP and F resulted in a dramatic increase in FKBP5 expression at the protein level in both the HESC cell line and ESCs isolated from fertile patients compared to the control ($p \leq 0.01$ and $p \leq 0.01$) and compared to cells treated with cAMP ($p \leq 0.01$ and $p \leq 0.001$) (figure 4.9 A and C). Treatment with F alone also resulted in a significant increase in FKBP5 expression, but unlike WT1, this was seen in both the HESC cell line and the primary ESCs compared to the control ($p \leq 0.001$ and $p \leq 0.05$).To further support the results obtained at the protein level using western blot analysis, qPCR was undertaken to see if the transcript levels reflected the translated protein levels.

It was found that qPCR analysis reciprocated the results seen at the protein level for both WT1 and FKBP5 expression (figure 4.10).

Treatment with cAMP alone resulted in a significant increase in the expression of WT1 compared to the control in both the HESC cell line and primary ESCs isolated from fertile patients ($p \leq 0.05$ and $p \leq 0.05$) (figure 4.10A). The addition of F along with cAMP resulted in a further significant increase in WT1 expression in the HESC cell line only compared to the control and also compared to cAMP treatment ($p \leq 0.01$ and $p \leq 0.05$). Treating the HESC cell line with only F also resulted in a significant increase in WT1 expression at the mRNA level compared to the control ($p \leq 0.05$). Co-treatment with cAMP and F resulted in no significant change in WT1 expression in the ESCs isolate from fertile patients.

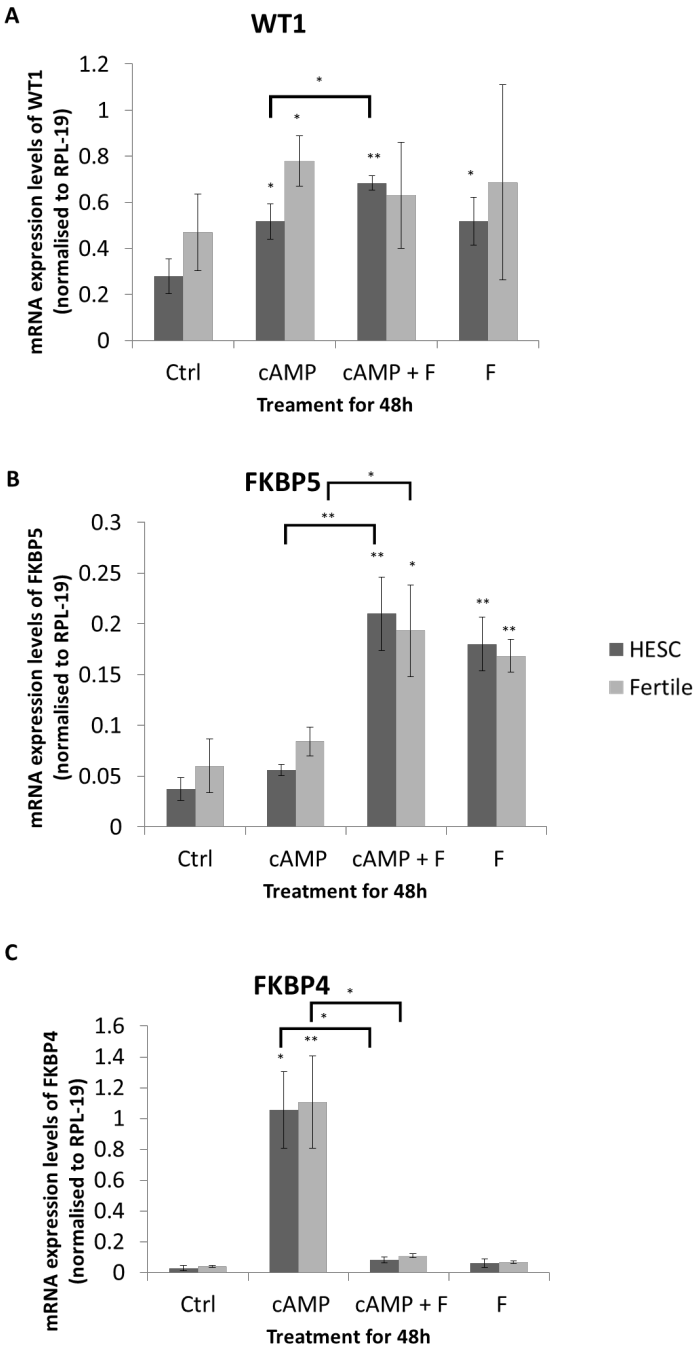


Figure 4.10 The effect of cortisol (F) on the mRNA expression levels of WT1 (A), FKBP5 (B) and FKBP4 (C) in the HESC cell line and ESCs isolated from fertile patients during decidual transformation. After 48 h of treatment, the HESC cells (n=3) and ESCs isolated from fertile patients (n=5) were lysed, RNA extracted and converted to cDNA and used for qPCR analysis. Cells underwent 48h of treatment with either cAMP, cAMP and F or F alone and compared to the control. qPCR analysis using gene specific primers was carried out to establish the expression levels at the mRNA level of WT1 (A), FKBP5 (B) and FKBP4 (C). Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

The gene expression levels of FKBP5 were undoubtedly significantly increased in both the HESC cell line and ESCs isolated from fertile patients treated with cAMP and F compared to the control ($p \leq 0.01$ and $p \leq 0.05$), with a 7 fold and 3 fold increase in expression respectively and also compared to cAMP treatment alone ($p \leq 0.01$ and $p \leq 0.05$) with a 3.75 fold and 2.4 fold increase in expression respectively (figure 4.10B).

In addition to FKBP5, another immunophilin; FKBP4 which is also crucial for successful implantation and decidualization was investigated to see whether cortisol exposure during decidual transformation had any effects on FKBP4 expression at the mRNA level only. It was found that cAMP treatment resulted in an increase in the expression of FKBP4 expression in both the HESC cell line ($p \leq 0.05$) and in the primary ESCs ($p \leq 0.01$) (figure 4.10C). Co-treatment with cAMP and F resulted in a dramatic decrease in the expression of FKPBP4 compared to the control in both the HESC cell line and primary ESCs ($p \leq 0.05$). Co-treatment with cAMP and F resulted in a 13 fold ($p \leq 0.05$) and 11 fold ($p \leq 0.05$) decrease in FKBP4 expression compared to cAMP treatment only (figure 4.10C). Treatment with F did not result in any significant alterations in the expression of FKBP4 in the primary ESCs compared to the control.

4.2.3 The effect of cortisone during decidual transformation of endometrial stromal cells

Following the investigation of the effect of cortisol and GR signalling during decidual transformation of the HESC cell line and ESCs isolated from fertile patients, it was decided to investigate the role of the biologically inactive glucocorticoid; cortisone. This would allow us to test whether the 11β-HSD1 dependent cortisol biosynthesis is modulating the expression of the key decidual markers with the stromal cells. It would also allow us to determine if the effects seen at both the protein and transcript level are due to the activation of the GR signalling pathway due to the presence of the active ligand; cortisol. Therefore, to explore the role of cortisone (compound E) and the GR signalling pathway on decidual transformation of the stromal cells, the HESC cell line and ESCs isolated from fertile patients were cultured and treated with the *in vitro* decidualization stimulus in combination with cortisone for 48 h.

Decidualization was determined based on the morphological appearance and changes in the stromal cells and by the expression of key decidual markers as seen previously. This comprises of a change in fibroblast shape cells to more rounded epithelial like cells and an induction of expression of dPRL and IGFBP-1.

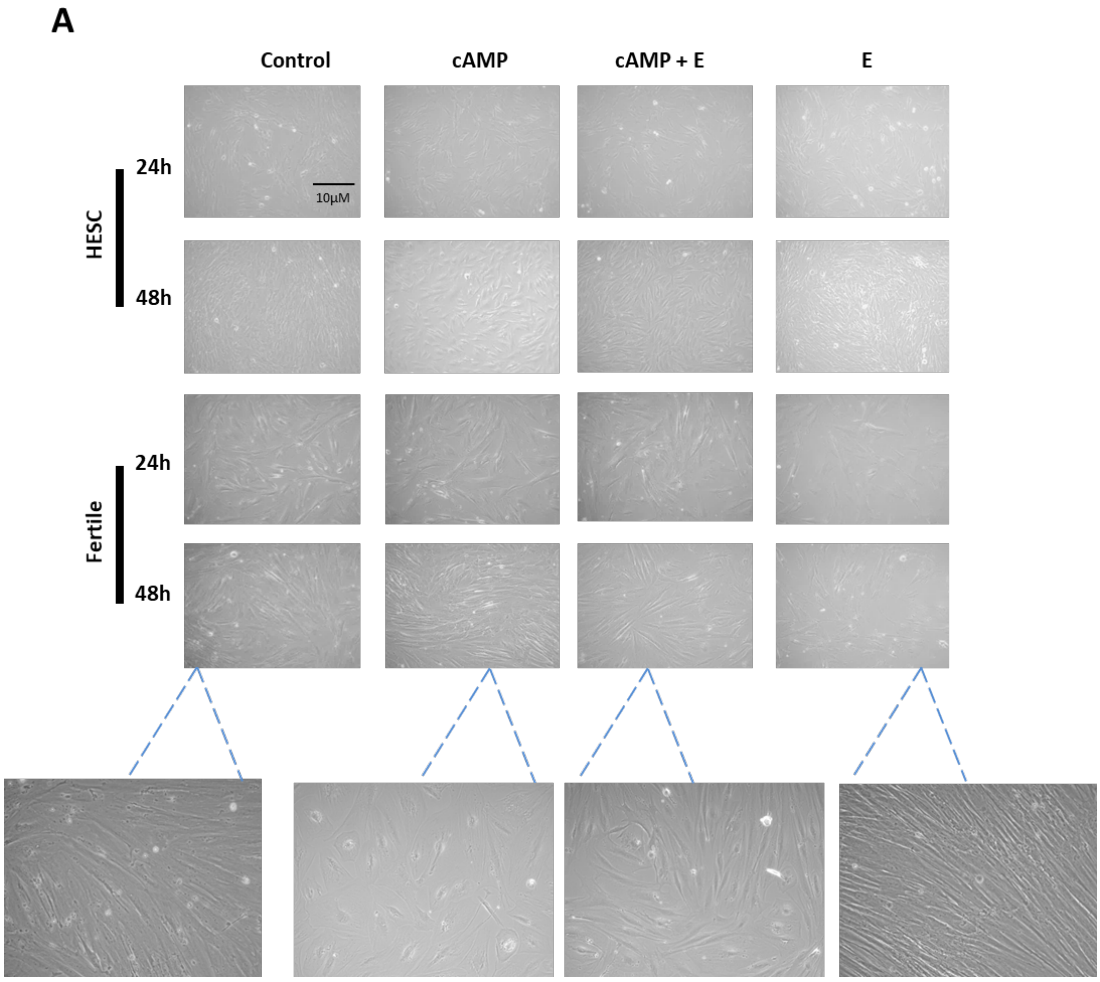
4.2.3.1 The effect of cortisone on cell morphology and the expression of key decidual markers during decidual transformation

Cell monolayers were treated for 48 h with wither cAMP, cAMP + cortisone (E) or E alone and compared to control cells. It was found that treatment with the *in vitro* decidualization stimulus; cAMP, caused a significant change in cell morphology as mentioned above, where cells adopted a more rounded epithelial like phenotype compared to their normal fibroblastic phenotype of the control cells (figure 4.11 A and B).

This visual change in morphology seen in figure 4.11A after cAMP treatment indicates that the stromal cells have undergone successful decidual transformation in vitro as previously mentioned. This was further confirmed quantitatively using Image J analysis, where it was found that the both the HESC cell line and ESCs isolated from fertile patients treated with cAMP for 48 h had a significant increase in circularity compared to the control ($p \leq 0.001$; $p \leq 0.01$). And similar to results seen with F, it was also demonstrated that co-treatment with cAMP and E caused a significant reduction in average cell circularity values in both the HESC cell line and ESCs isolated from fertile patients compared to those treated with cAMP alone ($p \leq 0.01$; $p \leq 0.05$).

However, co-treatment with cAMP and E still resulted in average circularity values which were significantly higher than the untreated cells in both the HESC cell line and primary ESCs ($p \leq 0.01$ and $p \leq 0.05$). These alterations in cell morphology can be clearly seen visually in figure 4.11A. Treatment with E alone caused no significant difference in the average circularity values compared to the control in both the HESC cell line and ESCs isolated from fertile patients, comparable to values seen with F treatment (figure 4.11).

To further support the morphological changes seen in these cells – the concentration of key secreted decidual markers were measured using commercially available ELISA kits.


A
Control
cAMP
cAMP + E
E
HESC
24h
48h
Fertile
24h
48h
10μM

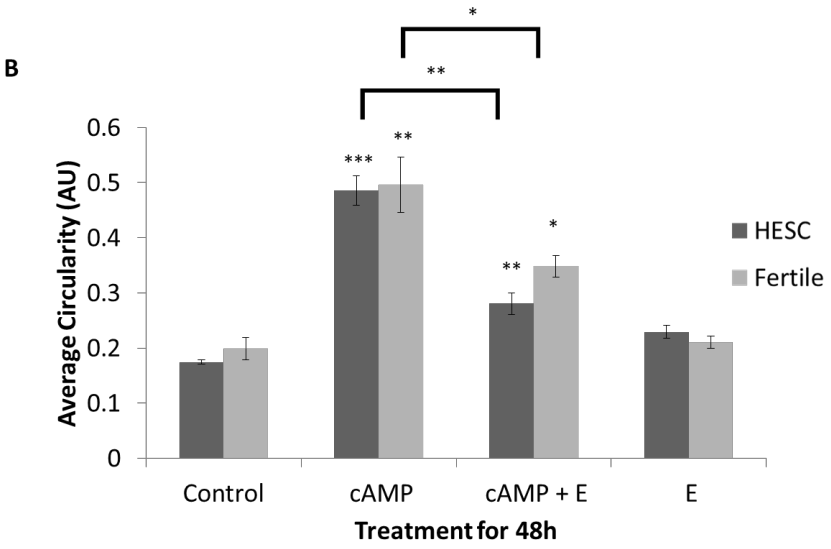

B
Average Circularity (AU)
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
cAMP
cAMP + E
E
Treatment for 48h
HESC
Fertile
*
**
***
**
**
*

Figure 4.11A and B. The effect of cortisone (E) on the morphology and circularity values of the HESC cell line and ESCs isolated from fertile patients during decidual transformation. A) Representative images taken of the cell monolayers after 48 h of treatments using inverted light microscopy (Zeiss) at a x10 objective. Scale bar indicates 10µM. Insets of representative images taken at a x20 objective are also shown for some treatment groups. These images were then used for Image J analysis to gain a quantitative measure of cell morphology. B) Thirty cells were chosen at random from each image taken of the HESC cell line (n=3) and ESCs isolated from fertile patients (n=5), therefore 90 cells were analysed from each treatment group for the HESC cell line and 150 cells were analysed from the ESCs isolated from fertile patients from each treatment group. Values are averaged from 90 cells (HESCs) and 120 cells (ESCs isolated from fertile patients) and bars indicate ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

It was found that treatment of both the HESC cell line and ESCs isolated from fertile patients with cAMP alone and co-treatment with cAMP and E caused a significant increase in the concentration of secreted prolactin compared to the control (p≤0.01 and p≤0.05; p≤0.01 and p≤0.01) (figure 4.12A). However, even though stromal cells co-treated with cAMP and F caused on average a decrease in the secretion of prolactin, there was no significant difference in these levels compared to cells treated with cAMP alone in both the HESC cell line and ESCs isolated from fertile patients.

Interesting results were obtained for secreted levels of another key decidual marker; IGFBP-1. It was found that treatment of the HESC cell line and ESCs isolated from fertile patients with cAMP alone caused a significant increase in the concentration of secreted IGFBP-1 compared to the control (p≤0.05 and p≤0.01), however unlike secreted prolactin, co-treatment with cAMP and E resulted in a further increase in the secreted levels of IGFBP-1 which was not statistically significant compared to cAMP treatment only (figure 4.12B), these results differ to that seen when treating the stromal cells with cAMP and F.

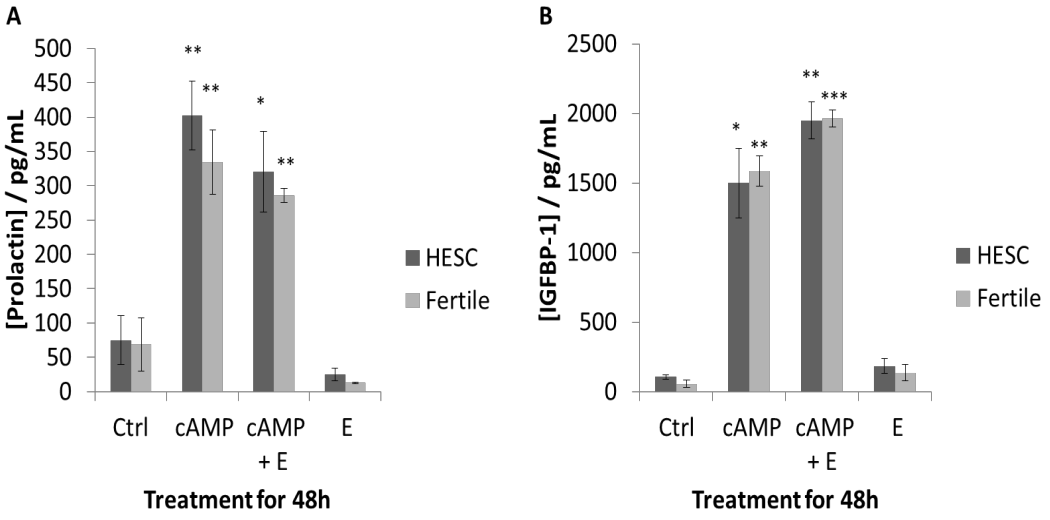


Figure 4.12. The effect of cortisone (E) on the levels of secreted decidual markers – prolactin (A) and IGFBP-1 (B) in the HESC cell line and ESCs isolated from fertile patients during decidual transformation. Media was extracted from the HESC cell line (n=3) and ESCs isolated from fertile patients (n=5) after 48 h of treatment with either cAMP, cAMP and E or E alone and compared to the control. ELISA experiments were carried out on the conditioned media to establish the concentration of key decidual proteins at the secreted level. Treatment with cAMP caused a significant increase in the secreted levels of both decidual markers compared to the control. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

These ELISA results were further confirmed using qPCR analysis to determine the expression levels of dPRL and IGFBP-1 at the mRNA level using gene specific primers (figure 4.13). Analysis of the results indicated that dPRL expression was induced significantly in HESCs and ESCs isolated from fertile patients treated with cAMP (p≤0.05 and p≤0.05). Similar to the secreted protein levels observed, treatment with cAMP and E resulted in a slight decrease in the average expression of dPRL at the mRNA level in the HESC cell line, but an increase in dPRL expression in primary ESCs (figure 4.13A). qPCR analysis to determine the expression levels of IGFBP-1 also supported the ELISA results obtained, where treatment with cAMP and co-treatment with cAMP and E resulted in an increase in IGFBP-1 expression in both the HESC cell line and primary ESCs (p≤0.05 and p≤0.01; p≤0.01 and p≤0.05) (figure 4.13B).

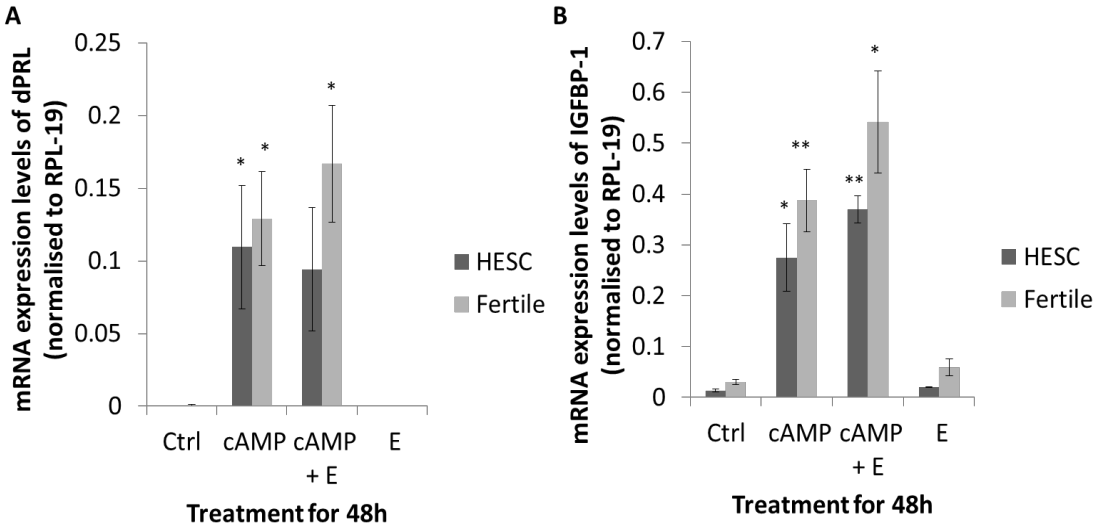


Figure 4.13 The effect of cortisone (E) on the mRNA expression levels of key decidual markers – prolactin (A) and IGFBP-1 (B) in the HESC cell line and ESCs isolated from fertile patients during decidual transformation. After 48 h of treatment the HESC cells (n=3) and ESCs isolated from fertile patients (n=5) were lysed, RNA extracted and converted to cDNA and used for qPCR analysis. Cells underwent 48h of treatment with either cAMP, cAMP and E or E alone and compared to the control. qPCR analysis was carried out to establish the expression levels at the mRNA level of dPRL (A) and IGFBP-1 (B). Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

4.2.3.2 The effect of cortisone on the expression of GR and 11β-HSD during decidual transformation

To test whether cAMP and E treatment were affecting the GR signalling pathway, it was decided that the expression of GR and the 11β-HSD enzymes during decidual transformation in the absence and presence of cortisone should be investigated. As previously described, the HESC cell line and ESCs isolated from fertile patients were cultured and treated with either cAMP, cAMP and E or E alone for 48 h and compared to the control.

The results obtained for the control cells and cells which have undergone *in vitro* decidualization have been previously described and will not be described here. Furthermore, it was found that co-treatment with cAMP and E resulted in a significant decrease in the expression of both GRα and GRβ in the HESC cell line compared to cells treated with only cAMP ($p \leq 0.01$ and $p \leq 0.01$) (figure 4.14A-C). This effect was also reciprocated in the ESCs isolated from fertile patients, however the average fold change in expression was not deemed to be significant. Interestingly, treatment with E alone also resulted in a decrease in the expression of both GR isoforms at the protein level compared to the control in the HESC cell line ($p \leq 0.01$). An overall decrease in GRα expression was also seen in the primary ESCs treated with E, but again this was not found to be statistically significant. Conversely, E treatment resulted in a slight increase in the average expression of GRβ in the ESCs isolated from fertile patients.

Remarkably, it was found that co-treatment with cAMP and E or E alone resulted in no significant alterations in the expression of 11β-HSD1 at the protein level either in the HESC cell line or ESCs isolated from fertile patients (figure 4.14 A and D. Whereas co-treatment with cAMP and E seems to dampen the average expression of the 11β-HSD2 at the protein level in both the HESC cell line and primary ESCs compared to cells treated with the decidualization stimulus only. This effect however was only deemed significant in the primary ESCs isolated from fertile patients ($p \leq 0.01$) (figure 4.14 A and E).

To further support the results obtained at the protein level using western blot analysis, qPCR was undertaken to see if the transcript levels reflected the translated protein levels.

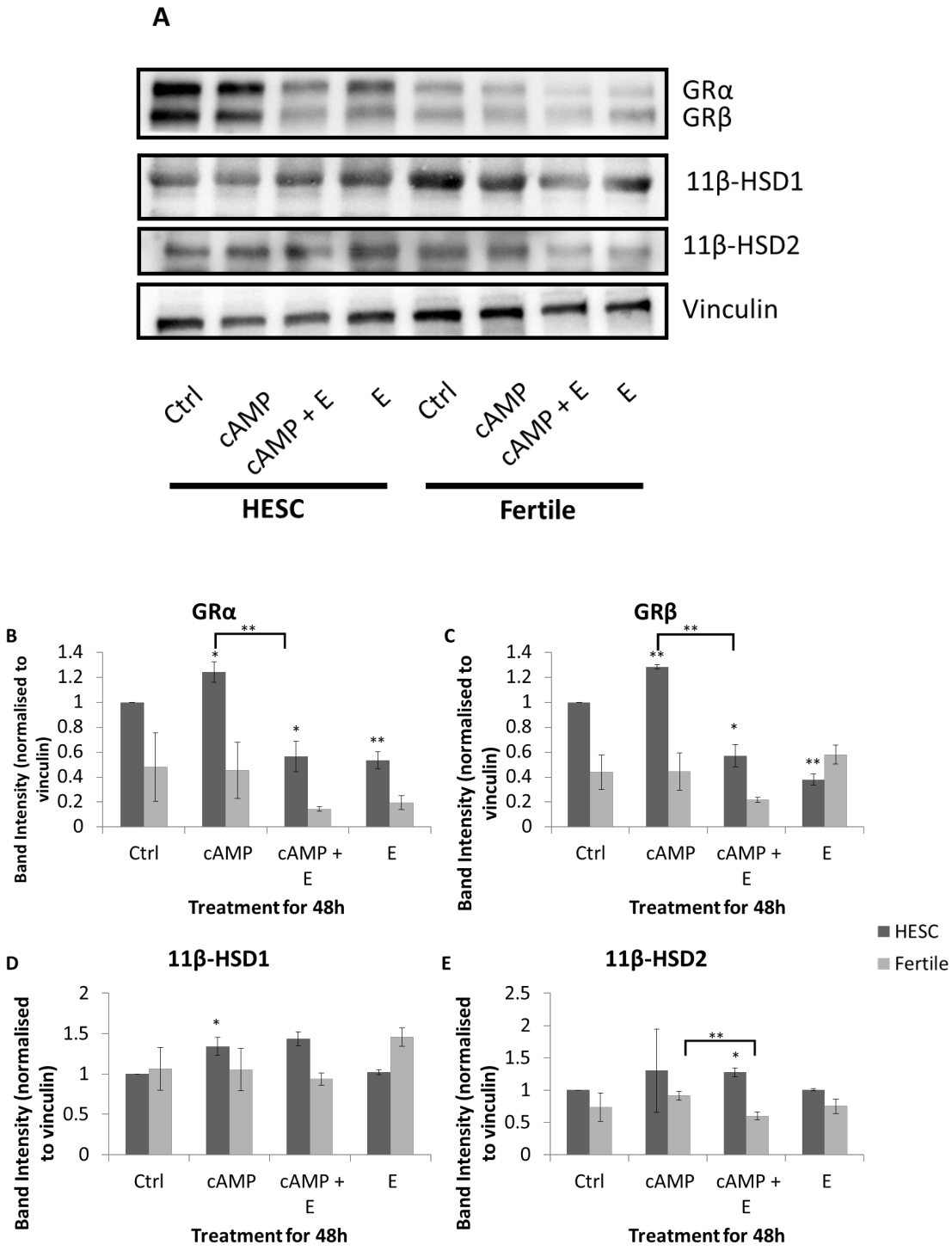


Figure 4.14 The effect of cortisone (E) on key proteins involved in the GR signalling pathway during decidual transformation in the HESC cell line and ESCs isolated from fertile patients. A) Representative images show the western blot analysis of GRα (95kDa), GRβ (90kDa), 11βHSD1 (32 kDa) and 11βHSD2 (40 kDa) protein levels in ESCs isolated from fertile patients (n=5) vs the HESC cell line (n=3) after 48h treatment with either cAMP, cAMP and E or E alone and compared to the untreated control. B-E) The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the house keeper protein; vinculin (124 kDa). Band intensity is displayed as the fold expression relative to the control HESC cells and normalised to the respective vinculin samples. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

qPCR analysis revealed that the mRNA expression levels of GR, 11β-HSD1 and 1β-HSD2 did not reciprocate the results seen at the protein level (figure 4.15). Poor correlations can be reported between the levels of mRNA and the levels of corresponding protein, which may be due to post-transcriptional modifications of the mRNA or post-translational modifications and management of the protein. Co-treatment with cAMP and E resulted in a decrease in the expression of GR in both the HESC cell line and ESCs isolated from fertile patients compared to the control ($p \leq 0.01$ and $p \leq 0.001$) (figure 4.15A) and compared to cells only treated with cAMP ($p \leq 0.05$ and $p \leq 0..05$). Treatment with E alone also resulted in a decrease in the expression of GR compared to the control ($p \leq 0.05$), but not to the same extent seen with the co-treatment. The same pattern of expression of GR was obtained in ESCs isolated from fertile patients, where GR transcript levels were also reduced by E treatment ($p \leq 0.05$).

Interestingly, qPCR analysis revealed that even though 11β-HSD1 mRNA levels were undoubtedly increased by cAMP treatment in both the HESC cell line and in the primary ESCs compared to the control ($p \leq 0.001$ and $p \leq 0.01$) (figure 4.15B), the addition of E along with cAMP caused an even further induction of the expression of 11β-HSD1 in both the HESC cell line and primary ESCs compared to cAMP treatment alone ($p \leq 0.01$ and $p \leq 0.05$). Whereas similar expression levels to the control were obtained when the ESCs were treated with E alone.

On the contrary, 11β-HSD2 expression is expressed at low levels during decidual transformation of both the HESC cell line and primary ESCs isolated from fertile patients as previously mentioned. However, the co-treatment with cAMP and E resulted in a further decrease in 11β-HSD2 expression levels compared to both the control cells ($p \leq 0.05$) and the cells treated with the in vitro decidualization stimulus only (not significant). This mRNA expression pattern is also seen in the ESCs isolated from the fertile patients, where co-treatment with cAMP and E resulted in a significant decrease in 11β-HSD2 expression levels compared to the cAMP treatment only ($p \leq 0.05$). The results obtained from qPCR analysis, particularly those for the primary ESCs confirm the results obtained at the protein level.

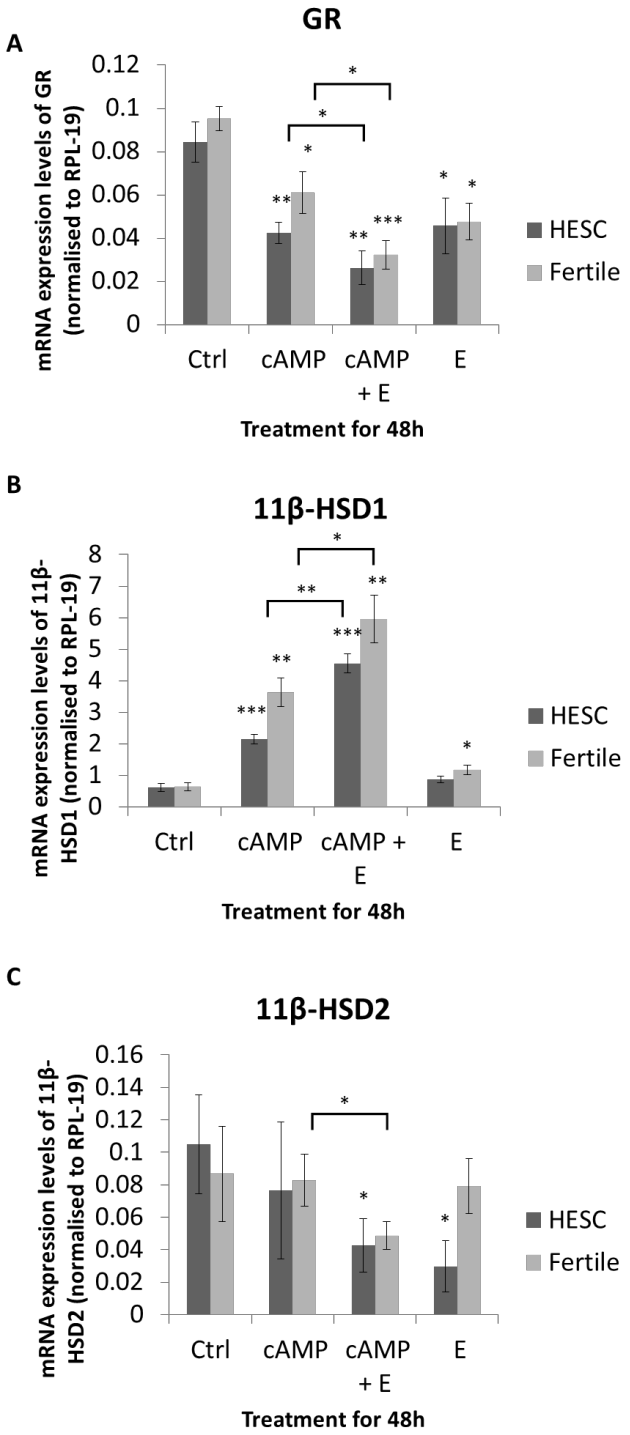


Figure 4.15 The effect of cortisone (E) on the levels of mRNA expression of GR (A), 11β-HSD1 (B) and 11β-HSD2 (C) in the HESC cell line and ESCs isolated from fertile patients during decidual transformation. After 48 h of treatment, the HESC cells (n=3) and ESCs isolated from fertile patients (n=5) were lysed, RNA extracted and converted to cDNA and used for qPCR analysis. Cells underwent 48 h of treatment with either cAMP, cAMP and E or E alone and compared to the control. qPCR analysis using gene specific primers was carried out to establish the expression levels at the mRNA level of GR (A) and 11β-HSD1 (B) and 11β-HSD2 (C). Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

4.2.3.3 The effect of cortisone on the expression of potential GR targets during decidual transformation

In addition to cortisol, it was decided to investigate the effect of cortisone on the expression of a selection of genes which are essential within the human endometrium particularly during decidualization. These include WT1, a known gatekeeper of decidualization and the immunophilins; FKBP4 and FKBP5. This would allow us to elucidate whether it is the biosynthesis and presence of the active ligand; cortisol due to the presence of 11β-HSD1 within the endometrium which is modulating the expression of these target proteins or whether this effect is independent of cortisol treatment.

Results for *in vitro* decidualization have been previously described and will not be discussed in this section. Western blot analysis and the consequent quantification of the protein bands obtained revealed that co-treatment of the HESC cell line with cAMP and E resulted in a significant decrease in the expression of WT1 at the protein level compared to the control ($p \leq 0.05$) and cAMP (not significant) (figure 4.16 A-B). On the contrary, ESCs isolated from fertile patients treated with both cAMP and E did not mirror this inhibition of WT1 expression, where co-treatment actually resulted in an increase in the expression of WT1 compared to both the untreated and cells treated with cAMP only (not significant). Protein levels of WT1 also differed between the HESC cell line and primary ESCs when treated with E alone, where E treatment in the HESC cell line caused no significant difference in the WT1 expression levels compared to the control but actually caused an induction in the expression levels of WT1 in the ESCs isolated from fertile patients compared to the control ($p \leq 0.05$) (figure 4.16 A-B).

Furthermore, it was found that co-treatment with cAMP and E and also treatment with E alone resulted in a dramatic increase in FKBP5 expression at the protein level in the HESC cell line only compared to the control ($p \leq 0.05$) and treatment with the decidualization stimulus; cAMP ($p \leq 0.01$) (figure 4.16A and C). As seen with WT1 expression levels, differing results were obtained for FKBP5 expression levels on the addition of E. Treatment of the primary ESCs isolated from fertile patients with cAMP and E and also E alone, did not result in any significant alteration of the expression of FKBP5 at the protein level (figure 4.16 A and C).

To further support the results obtained at the protein level using western blot analysis, qPCR was undertaken to see if the transcript levels reflected the translated protein levels.

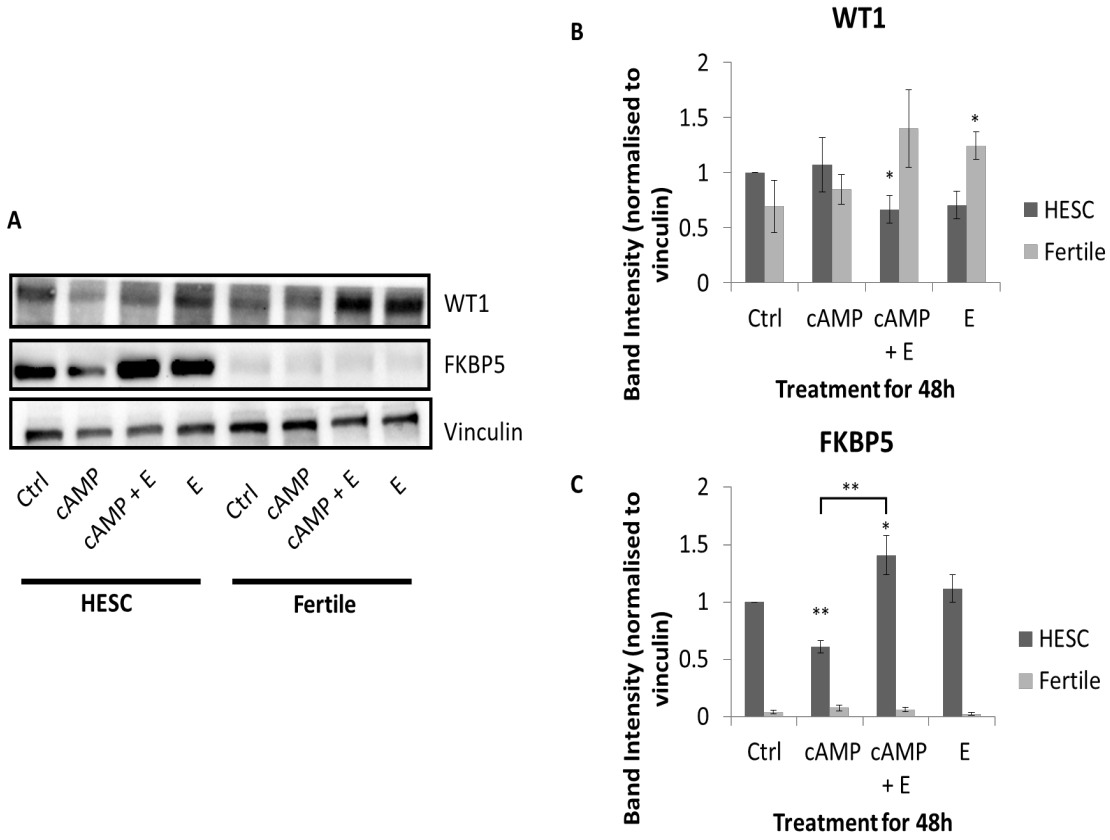


Figure 4.16 The effect of cortisone (E) on WT1 and FKBP5 protein expression during decidual transformation in the HESC cell line and ESCs isolated from fertile patients. A) Representative images show the western blot analysis of WT1 (52 kDa), FKBP5 (51 kDa) and vinculin (124 kDa) protein levels in ESCs isolated from fertile patients (n=5) vs the HESC cell line (n=3) after 48h treatment with either cAMP, cAMP and E or E alone and compared to the untreated control. B-C) The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the house keeper protein; vinculin. Band intensity is displayed as the fold expression relative to the control HESC cells and normalised to the respective vinculin samples. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

Unlike the results obtained when treating the ESCs with the in vitro decidualization and cortisol, the qPCR results obtained for the treatments with E in general did not reciprocate the results obtained at the protein level using western blot analysis and consequent band intensity quantification. The results obtained for cAMP treatment alone have been described previously and will therefore not be discussed in this section.

Using qPCR, it was found that the co-treatment of cAMP and E resulted in a significant increase in WT1 expression at the mRNA level compared to the control in both the HESC cell line and ESCs isolated from fertile patients (p≤0.05 and p≤0.01), which reflected the

results seen at the protein level for the primary ESCs only (figure 4.17A). Treatment with E alone resulted in no significant alteration in WT1 expression in either study group.

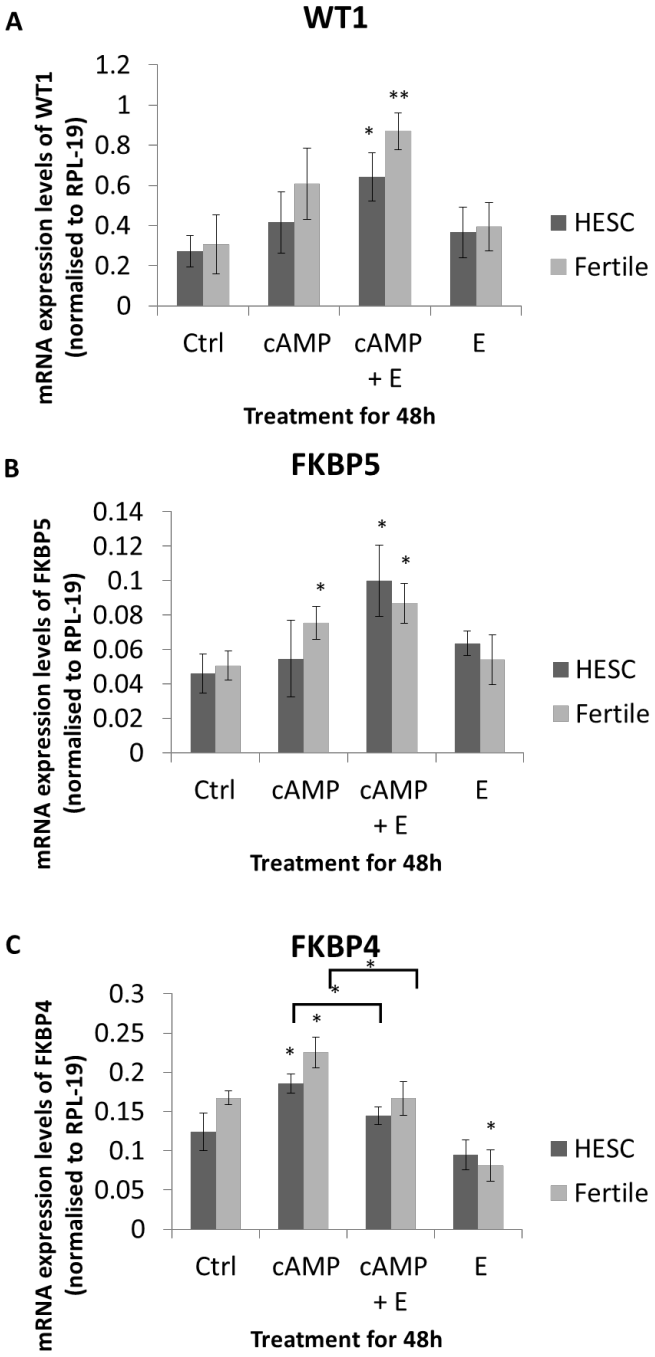


Figure 4.17 The effect of cortisone (E) on the mRNA expression levels of WT1 (A), FKBP5 (B) and FKBP4 (C) in the HESC cell line and ESCs isolated from fertile patients during decidual transformation. After 48h of treatment, the HESC cells (n=3) and ESCs isolated from fertile patients (n=5) were lysed, RNA extracted and converted to cDNA and used for qPCR analysis. Cells underwent 48 h of treatment with either cAMP, cAMP and E or E alone and compared to the control. qPCR analysis using gene specific primers was carried out to establish the expression levels at the mRNA level of WT1 (A), FKBP5 (B) and FKBP4 (C). Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ are considered significant.

It was seen in the western blot analysis, that FKBP5 protein expression was undeniably higher in the HESC cell line compared to the ESCs isolated from fertile patients. However, these results are not reproduced at the mRNA level as no significant difference in the expression levels of FKBP5 are observed between the HESC cell line and the primary ESCs (figure 4.17B). Co-treatment with cAMP and E resulted in a significant increase in FKBP5 expression at the mRNA level compared to the control in the both the HESC cell line and ESCs isolated from fertile patients ($p \leq 0.05$ and $p \leq 0.05$). Whereas, treatment with E alone did not cause any significant change in FKBP5 expression levels compared to the control in either study group.

And finally, it was found that co-treatment with cAMP and E resulted in a significant decrease in the expression of FKBP4 compared to cAMP treatment alone in both the HESC cell line and ESCs isolated from fertile patients ($p \leq 0.05$ and $p \leq 0.05$) but no significant change in expression compared to the untreated cells (figure 4.17C). Treatment with E alone resulted in a further decrease in FKBP4 expression at the mRNA level in both the HESC cell line and primary ESCs compared to the control and cAMP and E co-treatment, but was only considered significant in the case of the ESCs isolated from fertile patients compared to the untreated cells ($p \leq 0.05$). Western blot analysis was not carried out for FKBP4 expression levels due to the absence of a suitable antibody, therefore the transcript levels obtained here cannot be compared to any translated levels.

4.2.4 The effect of the GR antagonist; RU486 during decidual transformation of endometrial stromal cells

Due to the apparent effect of glucocorticoid exposure and the consequent activation of the GR signalling pathway within the ESCs and the effect on key proteins involved in decidual transformation, it was decided to determine whether antagonising GR would have any significant impact on the stromal cell morphology, expression of key decidual markers and also the expression of key proteins involved in the GR signalling pathway. The GR antagonist RU486 was used, which is also known as mifepristone. This would allow for the identification of GR dependent genes which are responsive to endogenous glucocorticoid exposure or the biosynthesis of the active ligand; cortisol in differentiating ESCs.

Firstly, experiments were conducted to optimise the time needed to treat the cells with the GR antagonist; RU486 for the ESCs to achieve a suitable level of GR down-regulation. Due to the promising results retrieved in this chapter so far, it was decided that optimisation would take place using the HESC cell line which is a suitable model for stromal cells isolated from fertile patients.

The HESC cell line were cultured and treated with RU486 in addition to cortisol (F) at varying time points and consequently the cells were lysed and protein extracted post-treatment. Western blot analysis revealed that 48 h was the optimum time period to treat the ESCs with, as this resulted in the lowest expression of GR, therefore indicating that the antagonist was successful in dampening down GR activation in these cells as it was able to bind to GR and block the receptor signalling pathway instead of activating it. Figure 4.18 demonstrates how the GR expression levels at the protein levels alter with varying incubation times with the antagonist; RU486 and cortisol (F) (figure 4.18).

Figure 4.18 A and B demonstrate that the 48h treatment with RU486 and F was the only time point to elicit a significant alteration in the expression levels of GR at the protein level, where there was a significant reduction in GR expression levels compared to the control (0 h) ($p \leq 0.01$). The expression levels of the sex steroid hormone receptors were also monitored to ensure that exposure to RU486 was not having any significant effect on their protein expression levels (figure 4.18 A and C-E).

Western blot analysis demonstrated that treatment with RU486 did not cause any significant alterations in the expression levels of both ER and AR at any of the time points studied (figure 4.18A and C, figure 4.18A and E respectively). Similar results were also obtained for PR; however, treatment with RU486 + F did seem to cause a significant reduction in the expression levels of PR after 0.5h of treatment compared to the control (0 h) ($p \leq 0.05$). This effect was not seen at any other time point, where 1h of treatment with RU486 showed no significant difference in PR expression compared to the control. These results initially seemed surprising as RU486 is a known anti-progestogen and therefore is known to have an effect on PR. However, it is known that RU486 is a progesterone antagonist and therefore block the actions of progesterone by blocking the PR. However as no progesterone was added in these experiments, this effect may not have been seen. Furthermore, additional time points and concentrations may also be needed if we are to see an effect of RU486 on PR expression *in vitro*.

Therefore, due to these results presented in figure 4.18, it was decided for the following experiments that the ESCs would be treated for 48h with RU486.

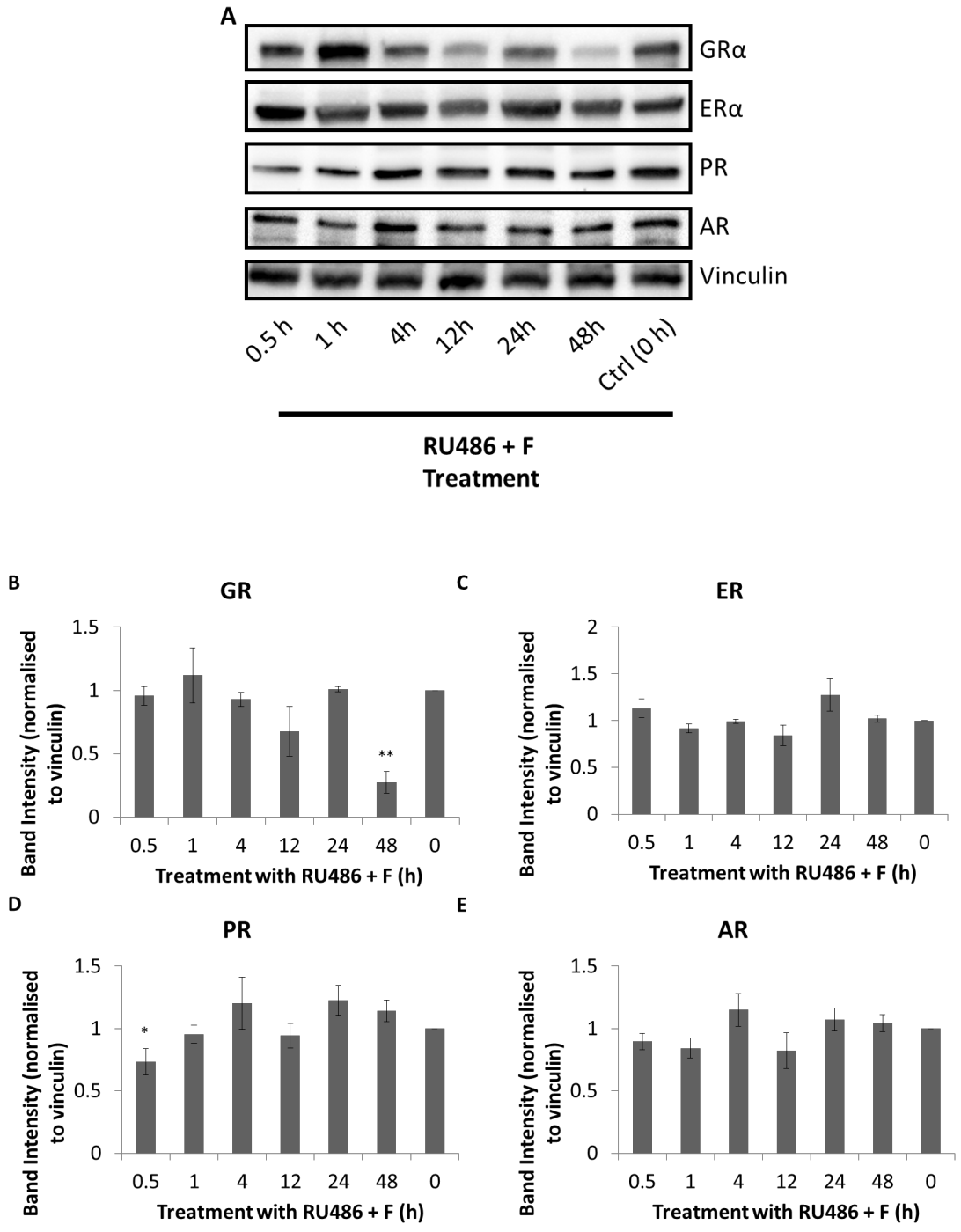


Figure 4.18 The effect of RU486 (mifepristone) and cortisol (F) on the protein expression of GR and the sex steroid hormone receptors; estrogen receptor (ER), progesterone receptor (PR) and the androgen receptor (AR) in the HESC cell line. A) Representative images show the western blot analysis of GRα (95 kDa), ER (66 kDa), PR (116 kDa), AR (110 kDa) and vinculin (124 kDa) protein levels in the HESC cell line (n=3) after varying incubation times with the GR antagonist; RU486 in addition to cortisol (F). B-E) The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the house keeper protein; vinculin. Band intensity is displayed as the fold expression relative to the control time point (0h) and normalised to the respective vinculin samples. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

4.2.5 The effect of the GR antagonist; RU486 and either cortisol or cortisone during decidual transformation of endometrial stromal cells

Due to the successful treatment of the HESC cell line with RU486, it was decided to treat both the HESC cell line and ESCs isolated from fertile patients with RU486 in combination with the stress hormone cortisol or cortisone in the presence and absence of the *in vitro* decidualization stimulus. This would aid us in identifying whether cortisol and the consequent activation of the GR signalling pathway or cortisone is affecting the cells morphology and biochemistry. Therefore, this next section will investigate the effect of antagonising the GR signalling pathway in ESCs during decidual transformation in the presence and absence of either cortisol or cortisone.

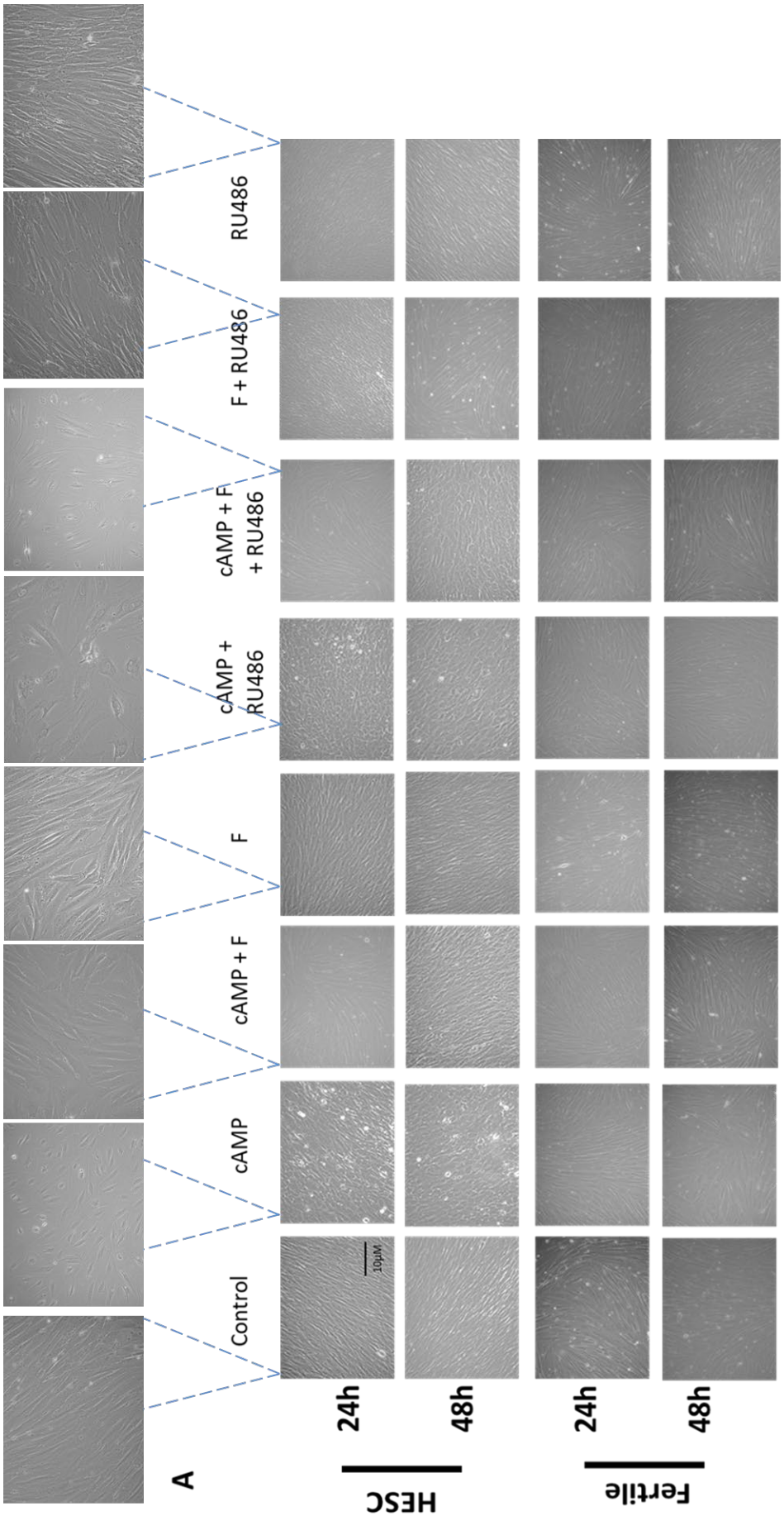
The HESC cell line and ESCs isolated from fertile patients were cultured and treated with the *in vitro* decidualization stimulus in combination with the stress hormone, cortisol or the inactive hormone cortisone, in the absence and presence of the GR antagonist; RU486 for 48h.

Decidualization was determined based on the morphological appearance and changes in the stromal cells as previously mentioned and by the expression of key decidual markers.

4.2.5.1 The effect of RU486 and cortisol on cell morphology and the expression of key decidual markers during decidual transformation

Cell monolayers were treated for 48 h with either cAMP, cAMP + cortisol (F), RU486, cAMP + RU486, cAMP + RU486 + F or F alone and compared to control cells. Results obtained for cells treated with cAMP and F has been described previously in this chapter and therefore will not be described in this section (figure 4.19 A and B).

It was found that co-treatment with the *in vitro* decidualization stimulus; cAMP and the GR antagonist; RU486 caused a significant change in the cell morphology in both the HESC cell line and in ESCs isolated from fertile patients compared to the control ($p \leq 0.001$ and $p \leq 0.001$) (figure 4.19A and B). Therefore the cells adopted a more rounded epithelial like phenotype compared to their normal fibroblastic phenotype. These results were comparable to the results obtained with cAMP treatment alone, which have been described previously. Therefore, this indicates that the stromal cells have undergone successful decidual transformation *in vitro* in the presence of both cAMP and RU486.


A
HESC
Fertile
24h
48h
24h
48h
Control
cAMP
cAMP + F
F
cAMP + RU486
cAMP + F + RU486
F + RU486
RU486
10μM

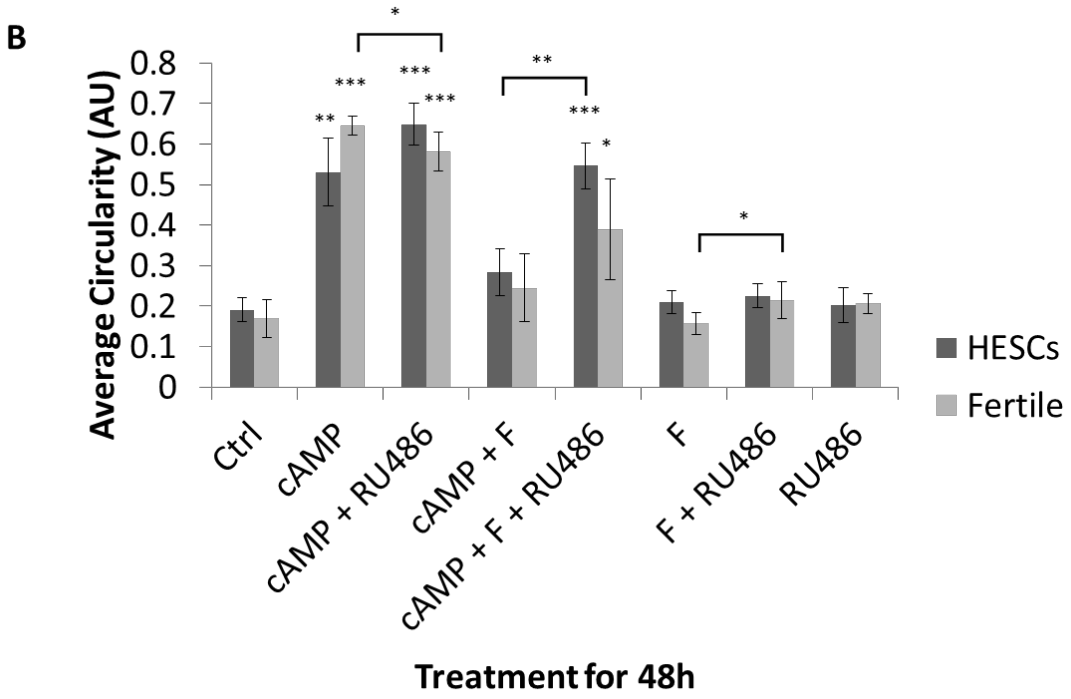


Figure 4.19A and 4.19B. The effect of cortisol (F) and the GR antagonist (RU486) on the morphology and circularity values of the HESC cell line and ESCs isolated from fertile patients during decidual transformation. A) Representative images taken of the cell monolayers after 48 h of treatments using inverted light microscopy (Zeiss) at a x10 objective. Scale bar indicates 10μM and applies to all images seen in this figure. These images were then used for Image J analysis to gain a quantitative measure of cell morphology. B) Thirty cells were chosen at random from each image taken of the HESC cell line (n=3) and ESCs isolated from fertile patients (n=5), therefore 90 cells were analysed from each treatment group for the HESC cell line and 150 cells were analysed from the ESCs isolated from fertile patients from each treatment group. Values are averaged from 90 cells (HESCs) and 120 cells (ESCs isolated from fertile patients) and bars indicate ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

Interestingly, as previously mentioned in this chapter, co-treatment with cAMP and F caused a significant reduction in average cell circularity values in both the HESC cell line and ESCs isolated from fertile patients compared to those treated with cAMP alone, where comparable average circularity values to the control cells were obtained. However the addition of RU486 in combination with cAMP and F resulted in an increase in the average circularity values of the HESC cells and ESCs isolated from fertile patients compared to the untreated control cells ($p \leq 0.001$ and $p \leq 0.05$). In addition, treatment with cAMP, F and RU486 also resulted in a significant increase in average circularity values of the HESC cell line compared to those treated cAMP and F only ($p \leq 0.01$), an overall increase in circularity values was obtained for the primary ESCs but was not deemed significant (figure 4.19B). These changes in morphology can be both seen visually (figure 4.19A) and quantitatively as a measure of circularity (figure 4.19B). Finally, treatment with F and RU486 or RU486 alone did not cause any significant changes in cell morphology using a measure of circularity compared to the control cells in both study groups.

To further support the morphological changes seen in these cells – the concentration of secreted decidual markers were measured using commercially available ELISA kits.

It was found that treatment of both the HESC cell line and ESCs isolated from fertile patients with cAMP and RU486 resulted in a significant increase in the secreted concentration of prolactin compared to the control ($p \leq 0.05$ and $p \leq 0.01$), which were comparable to cells treated with cAMP only (figure 4.20A). Interestingly, co-treatment with cAMP, F and RU486 caused a significant increase in the concentration of secreted prolactin compared to the control in both the HESC cell line and primary ESCs ($p \leq 0.01$ and $p \leq 0.01$). More importantly, treatment with cAMP, F and RU486 also caused a significant increase in the concentration of secreted prolactin compared to cells treated with cAMP and F only ($p \leq 0.05$ and $p \leq 0.05$), where the average concentration increased from approximately 236 pg/mL and 312 pg/mL to 465 pg/mL and 793 pg/mL in the HESC cell line and ESCs isolated from fertile patients respectively. These concentration levels obtained after stromal cells were exposed to all three treatments, were comparable to cells treated with cAMP only and those co-treated with cAMP and RU486 (figure 4.20A). Treatment with either RU486 alone or F and RU486 did not result in any changes in the levels of secreted prolactin compared to the control in either study groups.

On the contrary, the same effect was not shared with the concentration of secreted IGFBP-1. It was found that co-treatment with cAMP and RU486 resulted in a significant increase in the concentration of secreted IGFBP-1 compared to the control in both the HESC cell line and ESCs isolated from fertile patients ($p \leq 0.01$ and $p \leq 0.05$), comparable to the secreted levels of prolactin (figure 4.20B). However, co-treatment with cAMP and RU486 resulted in a significant decrease in the concentration of IGFBP-1 compared to cells treated with cAMP only in the HESC cell line only ($p \leq 0.05$). Additionally, it was found that treatment with the HESC cell line with cAMP, F and RU486 did cause a significant increase in the levels of secreted IGFBP-1 compared to the control ($p \leq 0.05$), but no differences in average levels of secreted IGFBP-1 were obtained for the stromal cells co-treated with cAMP and F in comparison to those treated with cAMP, F and RU486. Similarly to prolactin, treatment with either RU486 alone or F and RU486 did not result in any changes in the levels of secreted IGFBP-1 compared to the control in either study groups.

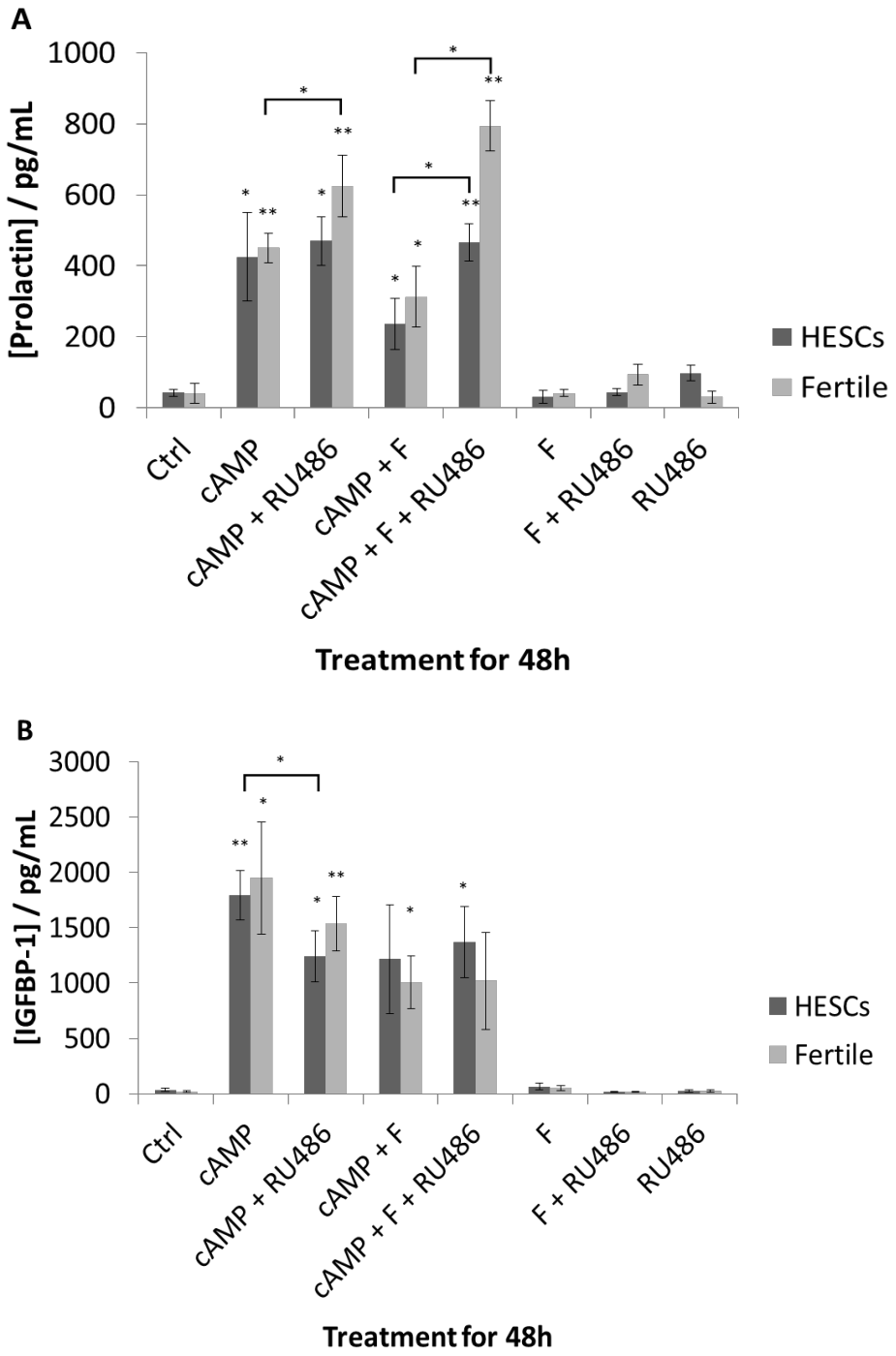


Figure 4.20. The effect of cortisol (F) and the GR antagonist (RU486) on the levels of secreted decidual markers – prolactin (A) and IGFBP-1 (B) in the HESC cell line and ESCs isolated from fertile patients during decidual transformation. Media was extracted after the HESC cell line (n=3) and ESCs isolated from fertile patients (n=5) underwent 48h of treatment with either cAMP, cAMP and RU486, cAMP and F, cAMP and F and RU486, F alone, F and RU486 or RU486 alone and compared to the control. ELISA experiments were carried out on the conditioned media to establish the concentration of key proteins at the secreted level. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

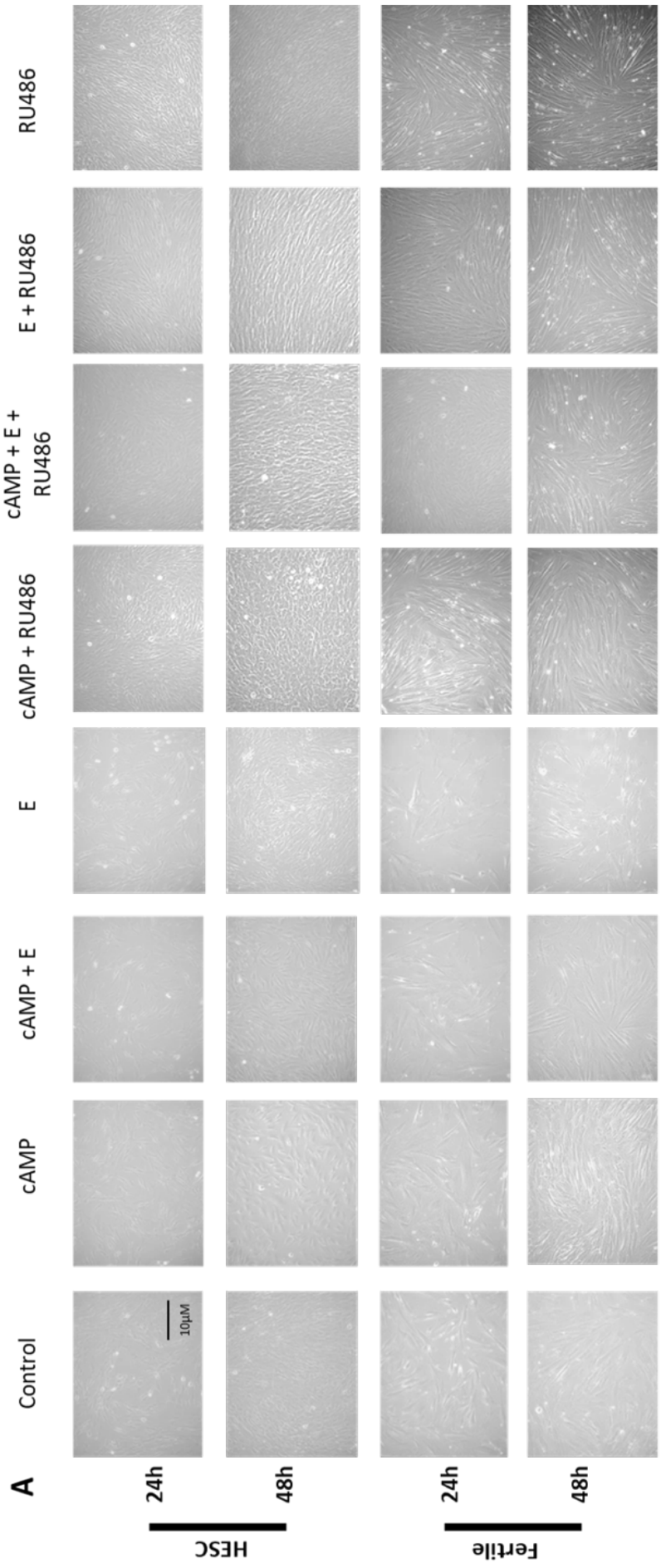
4.2.5.2 The effect of RU486 and cortisone on cell morphology and the expression of key decidual markers during decidual transformation

As previously mentioned, cell monolayers were treated for 48 h with either cAMP, cAMP + cortisone (E), RU486, cAMP + RU486, cAMP + RU486 + E or E alone and compared to control cells. Results obtained for cells treated with cAMP, cAMP + E, E, RU486 and cAMP + RU486 has been described previously in this chapter and therefore will not be described in this section (figure 4.21 A and B).

Results presented in section 4.2.5.1 indicate that co-treatment with cAMP and RU486 results in successful decidualization of the ESCs taking place and therefore will not be described here.

Interestingly, as previously mentioned in this chapter, co-treatment with cAMP and E caused a significant reduction in average cell circularity values in both the HESC cell line and ESCs isolated from fertile patients compared to those treated with cAMP alone, where comparable average circularity values to the control cells were obtained.

Treatment with cAMP, E and RU486 resulted in an increase in the average circularity values of the HESC cells and ESCs isolated from fertile patients compared to the untreated control cells ($p \leq 0.001$ and $p \leq 0.001$) (figure 4.21 A and B), which were analogous to the results seen when ESCs were treated with the active ligand cortisol (section 4.2.5.1). Comparable results were also obtained when comparing cells treated with cAMP, E and RU486 with those treated with cAMP and E only, where it was found that co-treatment with cAMP, E and RU486 resulted in a significant increase in average circularity values of the HESC cell line only compared to those treated with cAMP and E ($p \leq 0.001$) (figure 4.21 B), whereas an increase in the average cell circularity vales were observed in the primary ESCs – but this was not deemed significant (figure 4.21B). These changes in morphology can be both seen visually (figure 4.21A) and quantitatively as a measure of circularity (figure 4.21B). Finally, treatment with E and RU486 did not cause any significant changes in cell morphology using the measure of circularity compared to the control cells in both study groups (figure 4.21 B).


A
Control
cAMP
cAMP + E
E
cAMP + RU486
cAMP + E + RU486
E + RU486
RU486
10μM
24h
48h
24h
48h
HESC
Fertile

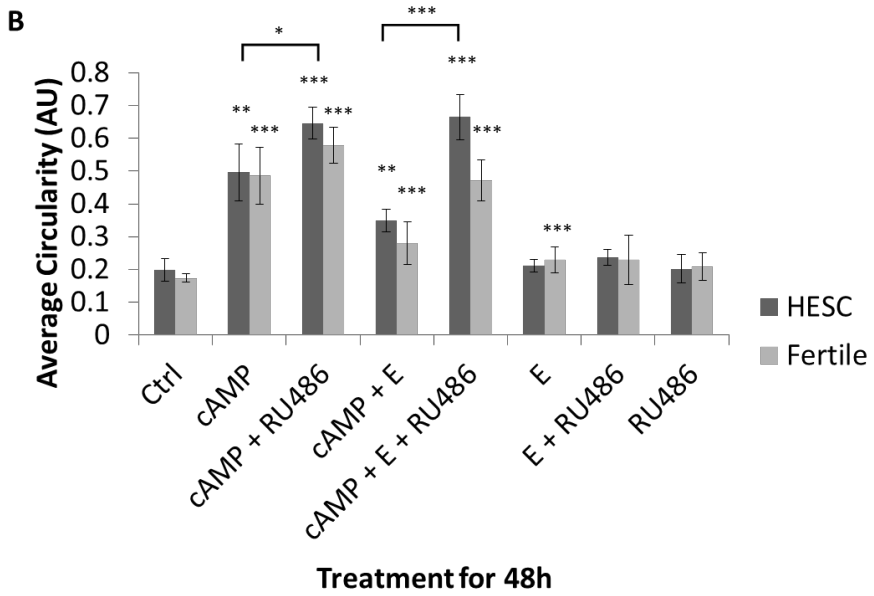


Figure 4.21A and 4.21B. The effect of cortisone (E) and the GR antagonist (RU486) on the morphology and circularity values of the HESC cell line and ESCs isolated from fertile patients during decidual transformation. A) Representative images taken of the cell monolayers after 48 h of treatments using inverted light microscopy (Zeiss) at a x10 objective. Scale bar indicates 10µM and applies to all images seen in this figure. These images were then used for Image J analysis to gain a quantitative measure of cell morphology. B) Thirty cells were chosen at random from each image taken of the HESC cell line (n=3) and ESCs isolated from fertile patients (n=5), therefore 90 cells were analysed from each treatment group for the HESC cell line and 150 cells were analysed from the ESCs isolated from fertile patients from each treatment group. Values are averaged from 90 cells (HESCs) and 120 cells (ESCs isolated from fertile patients) and bars indicate ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

To further support the morphological changes seen in these cells – the concentration of secreted decidual markers were measured using commercially available ELISA kits as described previously.

Results obtained for stromal cells treated with cAMP and RU486 and also RU486 only have been previously described in section 4.2.5.1 and will not be discussed here. It was found that treatment of both the HESC cell line and ESCs isolated from fertile patients with cAMP, E and RU486 resulted in a significant increase in the secreted prolactin levels compared to the control (p≤0.01), which reciprocated the results where cells were treated with the active ligand; F instead of E. And similarly to results obtained with F, treatment with cAMP, E and RU486 caused a significant increase in the secreted prolactin levels compared to cells treated with cAMP and E only in both study groups (p≤0.05) (figure 4.22 A). Treatment with E and RU486 did not result in any significant changes in prolactin secretion in either the HESC cell line or primary ESCs.

As described in section 4.2.5.1 figure 4.20B, the expression levels of prolactin were not mutual in regards to the concentration of secreted IGFBP-1. Results obtained for stromal cells treated with cAMP and RU486 and also RU486 only have been previously described in section 4.2.5.1 and will not be discussed here. However it was found that treatment of the HESC cell line and primary ESCs with cAMP, E and RU486 resulted in an increase in levels of secreted IGFBP-1 compared to the control ($p \leq 0.01$) (figure 4.22 B). However, unlike the prolactin results, it was found that treating the stromal cells with cAMP, E and RU486 actually resulted in an average overall decrease in IGFBP-1 expression levels compared to cells treated with cAMP and E only, which was deemed statistically significant for the HESC cell line only ($p \leq 0.05$). It must also be noted that in the HESC cell line, treatment with RU486 resulted in a significant decrease in the secreted levels of IGFBP-1 compared to the control ($p \leq 0.05$) and co-treatment with E and RU486 resulted in a significant decrease in the secreted levels of IGFBP-1 compared to stromal cells treated with only E ($p \leq 0.05$) (figure 4.22 B). This effect was not seen when the secreted levels of prolactin were investigated.

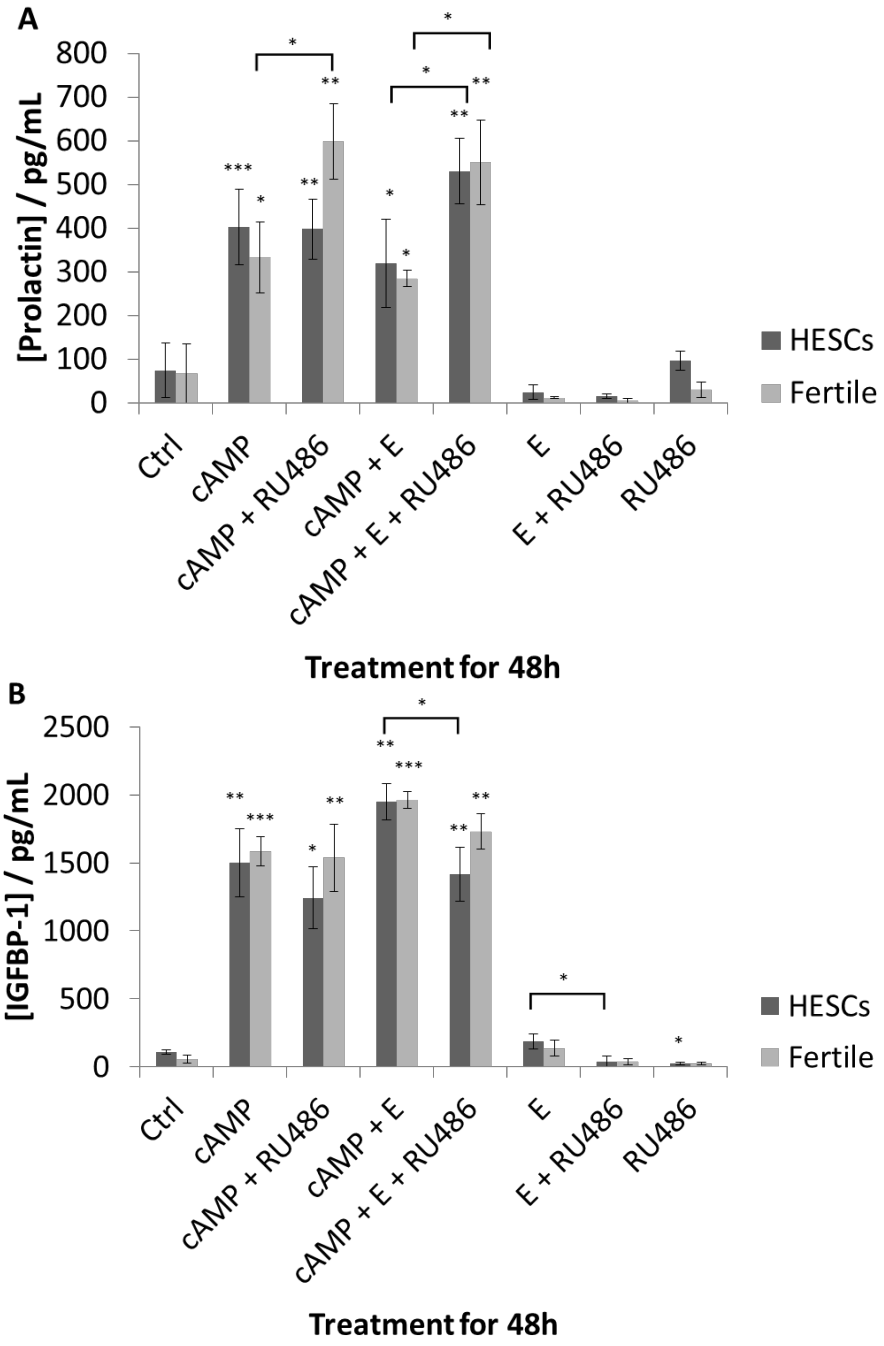


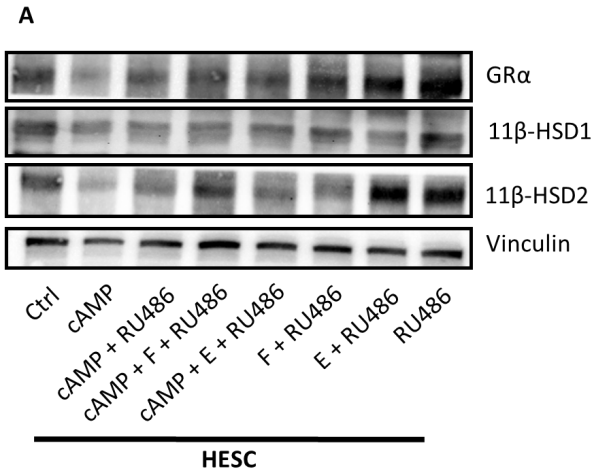
Figure 4.22. The effect of cortisone (E) and the GR antagonist (RU486) on the levels of secreted decidual markers – prolactin (A) and IGFBP-1 (B) from the HESC cell line and ESCs isolated from fertile patients during decidual transformation. Media was extracted after the HESC cell line (n=3) and ESCs isolated from fertile patients (n=5) underwent 48 h of treatment with either cAMP, cAMP and RU486, cAMP and E, cAMP, E and RU486, E alone, E and RU486 or RU486 alone and compared to the control. ELISA experiments were carried out on the conditioned media to establish the concentration of key proteins at the secreted level. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ are considered significant.

4.2.5.3 The effect of RU486 and cortisol or cortisone on the expression of GR and 11β HSD during decidual transformation

Due to the significant effects seen on both the cell morphology and the expression and secretion of key decidual markers when ESCs were treated with the GR antagonist; RU486 during decidual transformation in both the presence and absence of the stress hormone cortisol, it was decided to investigate the effect of RU486 and cortisol on the expression of GR and the 11β-HSD enzymes during decidual transformation. In addition to cortisol, the ESCs were also treated with the biologically inactive ligand; cortisone (E).

As previously mentioned, the HESC cell line and the primary ESCs isolated from fertile patients were cultured and treated with either cAMP, cAMP + RU486, cAMP + F, cAMP + F + RU486, F, F + RU486 or RU486 alone for 48h and compared to the control. Furthermore, the cells were also treated with cAMP + E, cAMP + E + RU486 and E + RU486.

Immunoblot analysis was carried out to determine the protein expression levels of GR, 11β-HSD1 and 11β-HSD2 in the HESC cell line to further understand the role of antagonising GR in the presence of the *in vitro* decidualization stimulus in the presence and absence of the glucocorticoids; cortisol (F) and cortisone (E). Interestingly it was found that 48h co-treatment with cAMP + RU486 or cAMP + F + RU486 did not result in any significant changes in the expression of GR in the HESC cell line compared to the control cells (figure 4.23 A and B). However, treating the HESC cell line with cAMP, E + RU486 resulted in a significant decrease in the expression of GR at the protein level compared to the control ($p \leq 0.05$). The immunoblot images also suggest that treatment with F + RU486, E + RU486 and RU486 results in an increase in the expression of GR at the protein level, but after further analysis this was not deemed to be statistically significant (figure 4.23 A and B).



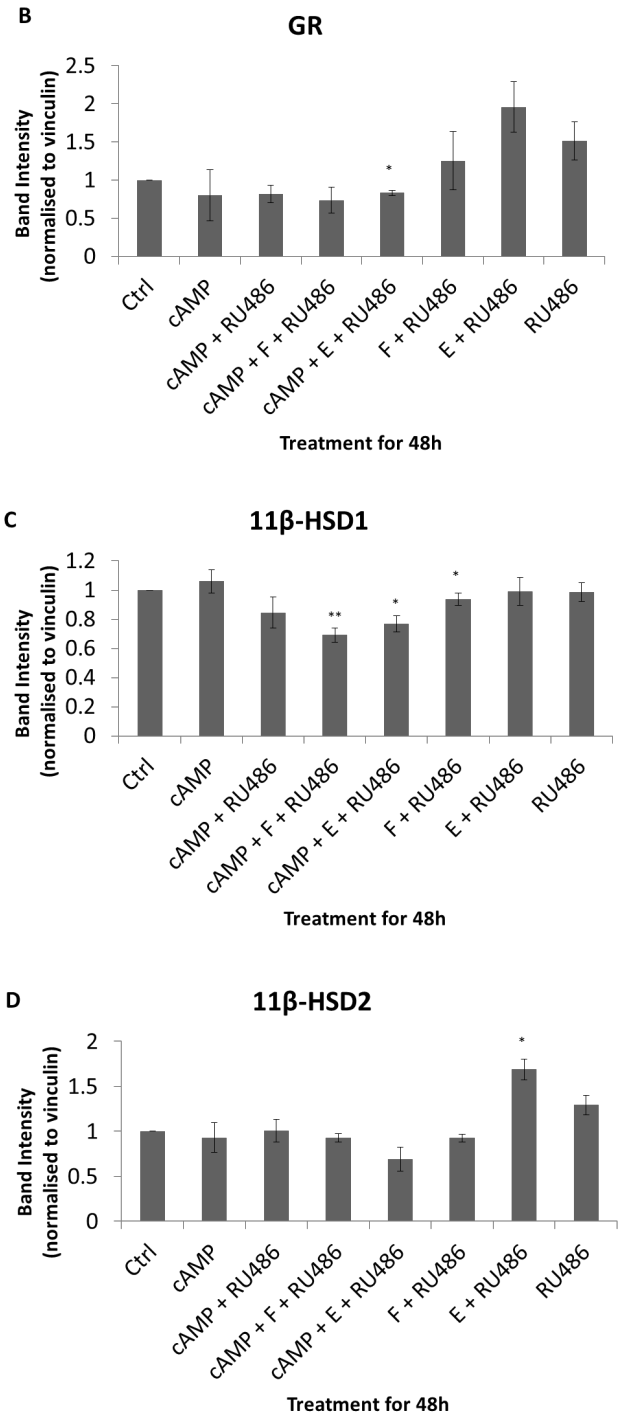
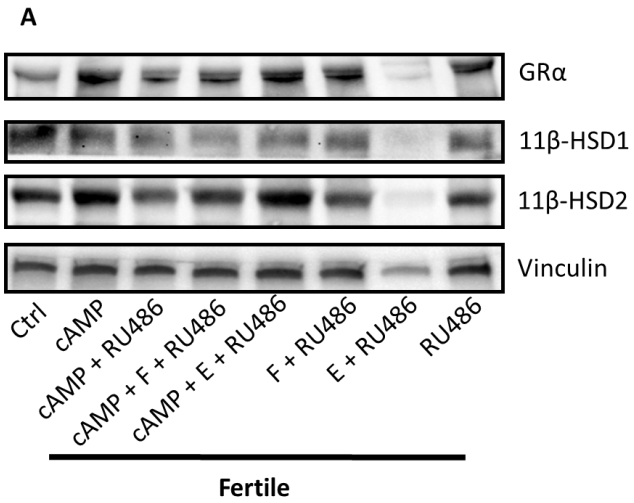


Figure 4.23 The effect of RU486 (or mifepristone) and cortisol (F) or cortisone (E) on the expression of GR the 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes type 1 and type 2 in the HESC cell line. A) Representative images show the western blot analysis of GRα (95 kDa), 11β-HSD1 (32 kDa), 11β-HSD2 (40 kDa) and vinculin (124 kDa) protein levels in the HESC cell line (n=3) after 48h treatment with either cAMP only, cAMP + RU486, cAMP + F + RU486, cAMP + E + RU486, F + RU486, E + RU486 and RU486 only compared to the untreated control. B-D) The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the house keeper protein; vinculin. Band intensity is displayed as the fold expression relative to the control cells and normalised to the respective vinculin samples Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

Subsequently, the protein expression levels of both 11β-HSD isoforms were investigated using immunoblots (figure 4.23 A, C and D). It was found that 48h co-treatment with cAMP and RU486 had no significant effect on the expression of 11β-HSD1 (figure 4.23 A and C) or 11β-HSD2 (figure 4.23 A and D). Whereas co-treatment with cAMP, F and RU486 resulted in a significant decrease in the protein expression levels of the type 1 isoform compared to the control ($p \leq 0.01$) which was not reciprocated when investigating the type 2 isoform. Treatment with cAMP, E and RU486 also resulted in a significant reduction in the expression of 11β-HSD1 compared to the control cells ($p \leq 0.05$), and similarly this treatment combination also resulted in a decrease in the expression of 11β-HSD2 at the protein level which was not statistically significant. Interestingly, treating the HESC cell line with F and RU486 for 48h also resulted in a decrease in the expression levels of 11β-HSD1 only compared the control ($p \leq 0.05$). Whereas treatment with E and RU486 had no effect on 11β-HSD1 expression, but resulted in a significant increase in the expression of 11β-HSD2 isoform compared to the control cells ($p \leq 0.05$). And finally, treatment with RU486 only resulted in no significant alterations in the expression of either isoform compared the untreated cells (figure 4.23 A, C and D).

Preliminary studies were also carried out to investigate whether similar results as above could be retrieved using primary ESCs isolated from fertile patients (n=2) (figure 4.24). The same treatment groups were used and immunoblot analysis was carried out after protein had been extracted from the cell monolayers.



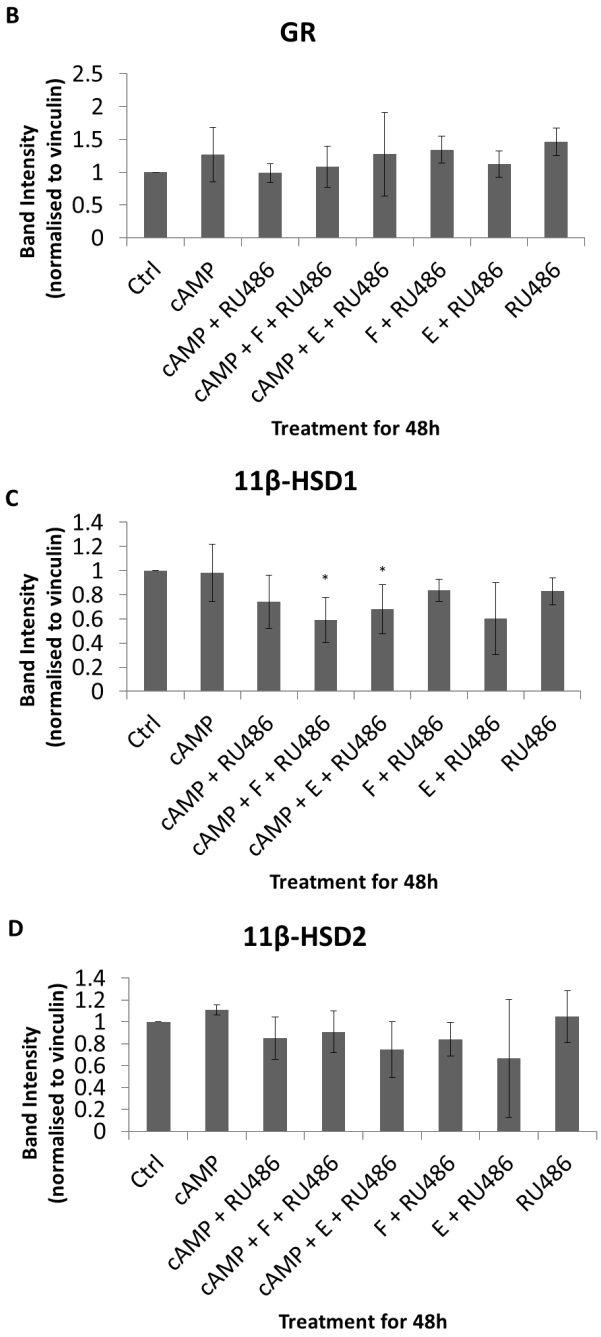


Figure 4.24 The effect of RU486 (mifepristone) and either cortisol (F) or cortisone (E) on the protein expression of GR and the 11β-hydroxysteroid dehydrogenase (11β-HSD) type 1 and type 2 enzymes in ESCs isolated from fertile patients. A) Representative images show the western blot analysis of GRα (95 kDa), 11β-HSD1 (32 kDa), 11β-HSD2 (40 kDa) and vinculin protein levels in ESCs isolated from fertile patients (n=2) after 48h treatment with either cAMP only, cAMP + RU486, cAMP + F + RU486, cAMP + E + RU486, F + RU486, E + RU486 and RU486 only compared to the untreated control. B-D) The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the house keeper protein; vinculin. Band intensity is displayed as the fold expression relative to the control cells and normalised to the respective vinculin samples. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

Even though similar results were obtained using the immunoblot analysis to study the protein expression of GR, 11β-HSD1 and 11β-HSD2 in primary ESCs isolated from fertile patients with the various treatment regimens. After densitometry band intensity and statistical analysis had been carried out, it was found that there was no significant alterations with any treatment group on the protein expression levels of GR (figure 4.24 A and B), 11β-HSD1 (figure 4.24 A and C) or 11βHSD1 (figure 4.24A and D). Therefore, these experiments need to be conducted on additional patient samples to truly understand if the results seen with the HESC cell line are also reciprocated in ESCs isolated from fertile patients.

4.2.6 The analysis of the GR promoter and the mechanism of regulation of GR expression and its targets

The observations presented in this chapter thus far, indicate that GCs and the GR signalling pathway may have a significant effect on the morphology of ESCs during decidual transformation. Additionally, key genes involved in the decidual response seem to be regulated by the actions of the GR signalling pathway. It was therefore decided to investigate the potential direct regulation of key target genes by GR using the HESC cell line. The HESC cell line was used due to insufficient cell numbers from primary biopsies and it was deemed to be a successful model of fertile endometrium in the investigation of the GR signalling pathway.

Initially promoter analysis using the TRANSFAC database was used to identify potential transcription factor (TF) binding sites in the promoter regions of target genes. Once potential binding sites were identified – chromatin immunoprecipitation (ChIP) was conducted to identify the presence of GR which is a TF, on the promoters of potential target genes in the HESC cell line, which has been shown to be an ideal model for the fertile endometrium.

4.2.6.1 Binding sites in the GR promoter region

The promoter sequence for the NR3C1 gene were retrieved and consequently run through the online TRANSFAC database using match analysis.

Characterisation of the NR3C1 promoter using the TRANSFAC database has successfully revealed the presence of multiple potential transcription factor binding sites under investigation (table 4.1). These included Activating protein 1 (AP-1), cAMP-responsive

element binding protein (CREB), WT1, and hormone response elements such as GR, PR and AR sites amongst others (data not shown).

TRANSFAC identified that the NR3C1 promoter (of 4000 bp) contains potential binding sites for the TF; CREB and its complexes. For example two binding sites were identified for cAMP-responsive element binding protein (between -458/-478bp and -487/-507bp) with a sequence similarity score of 0.906 and 0.870 respectively, indicating the similarity between the input sequence and the identified matched sequence for the TF binding site (table 4.1). Interestingly, the NR3C1 promoter also contains potential binding sites for GR (-3340/-3358bp), PR (-2383/-2401bp and -2695/-2713bp) and AR (-3196/-3214bp), all with sequence similarity scores of ≥ 0.851. In addition to these previously mentioned sites, the NR3C1 promoter also contains six potential binding sites for WT1which are all located within a 600bp section of the NR3C1 promoter (all 6 sites located between: -1011/-1606bp, all with a sequence similarity score of ≥ 0.959 (table 4.1).

		Promoter Position		
Transcription Factor	**Sequence**	**From (-bp)**	**To (-bp)**	**Sequence Similarity Score**
cAMP- responsive element binding protein (CREB)	aacattTGATgttcaggccat	-507	-487	0.870
	actggaTGACctaacaaactt	-478	-458	0.906
Wilms Tumour Suppressor	gggcgCGGGggagggtggg	-1031	-1013	0.982
	gagggTGGGggacggtcgg	-1049	-1031	0.988
	tggggCGGGggacggtcgg	-1606	-1588	0.959
	aggggTGGGggttgaactt	-1119	-1101	0.977
	gcgcgggGGAGggtgggtt	-1029	-1011	0.992
	ttgggcgGGAGgggtgggg	-1128	-1020	0.972
Glucocorticoid Receptor binding site, IR3 sites	taaGCACatattgtaagta	-3358	-3340	0.851
Progesterone receptor binding site, IR3 sites	aggtcaagcagTGTTctca	-2401	-2383	0.890
	atggggagatgTGTTctat	-2713	-2695	0.887
Androgen receptor binding site, IR3 sites	tgtaagcctgtttCTCCtca	-3214	-3196	0.904

Table 4.1 Table showing potential transcription factor binding sites on NR3C1 promoter. The online TRANSFAC database was used to identify potential transcription factor (TF) binding sites in the target promoter sequence. Match analysis was performed, which uses positional weight matrices (preferred nucleotide patterns) determined from TRANSFAC's broad compilation of experimental and theoretical determined binding sites to search a DNA sequence for potential TF binding sites. This table shows the results retrieved after TRANFAC analysis, where the NR3C1 promoter had potential binding sites for cAMP-responsive element binding proteins, WT1, GR, PR and AR amongst others. This table demonstrates the TF under investigation, the binding site sequence, the position of the potential binding site within the promoter sequence and the sequence similarity score between the input promoter sequence and the known sequence of the TF.

This output suggests that due to the presence of specific sequences within the NR3C1 promoter, GR could potentially be regulated by these transcription factors which can lead to transcriptional activation or repression. However it must be noted that individual binding sites within the promoter are not sufficient enough to indicate direct transcriptional function, therefore functional assessment of the binding sites needs to be carried out using alternative tools.

4.2.6.2 Potential regulation of key target genes by GR

After analysing the NR3C1 promoter, potential GR target gene promoters were also characterised using the TRANSFAC database. Preliminary analysis was carried out on the following genes: PRL, NR3C1, PGR, HSD11B2, HSD11B1, AR, WT1, FKBP5, FKBP4, ESR1, STAT4 and STAT5A. No previous work in this chapter has been conducted on STAT4 or STAT5a, but these genes were included due to their potential role within decidualization of the human endometrium (Okada et al, 2014). After identifying and retrieving the gene promoter sequences, it was investigated whether there were confirmed or potential binding sites for the following transcription factors; GR, AR, WT1, and PGR (table 4.2).

Promoter analysis was able to determine whether there were potential binding sites present within the input sequence or if there were confirmed binding sites present (table 4.2). It was revealed that all promoters under investigation either had validated binding sites or predicted binding sites for GR, AR, PGR and WT1 (table 4.2). Due to these promising preliminary results, it was decided to do a further in-depth analysis of the promoter regions of genes which have been investigated using molecular techniques previously in this chapter. Therefore the TRANSFAC online database was used to elucidate whether there were GR binding sites within the promoter regions of NR3C1, PRL, PGR, AR, HSD11B1, HSD11B2, WT1, FKBP5 and FKBP4. No further analysis was carried out on ESR1, STAT5A or STAT4 promoter regions due to no molecular work being carried out on these genes in this study.

Analysis of these target gene promoters for potential GR binding sites would shed light on whether GR could act as a TF and bind directly to these promoters and alter transcriptional activity.

The TRANSFAC database identified glucocorticoid receptor response elements in all promoters under investigation. These results are summarised in table 4.3 and table 4.4.

Consequently, the analysis retrieved using the TRANSFAC online database was used to further investigate the potential binding effects of GR onto its target promoters using the binding sites identified.

Symbol	GeneID	GR	AR	WT1	PGR
PRL	5617	- / + / +	- / + / +	- / + / +	- / + / +
NR3C1	2908	+ / + / +	- / + / +	- / + / -	- / + / +
PGR	5241	- / + / +	- / + / +	- / + / +	- / + / +
HSD11B2	3291	+ / + / +	- / + / -	- / + / -	- / + / -
HSD11B1	3290	- / + / +	- / + / +	- / + / -	- / + / +
AR	367	- / + / +	- / + / +	- / + / +	- / + / +
WT1	7490	- / + / -	- / + / +	- / + / -	- / + / +
FKBP5	2289	+ / + / +	- / + / +	- / + / -	- / + / +
FKBP4	2288	+ / + / +	- / + / +	- / + / -	- / + / +
ESR1	2099	+ / + / +	- / + / +	- / + / +	- / + / +
STAT4	6775	+ / + / -	- / + / +	- / + / -	- / + / -
STAT5A	6776	- / + / +	- / + / +	- / + / +	- / + / +

Legend	
+ / + / +	TF has a validated binding site and has at least 1 interaction evidence(s) with the respective gene
- / + / +	TF has a predicted binding site and has at least 1 interaction evidence(s) with the respective gene
+ / + / -	Less than 1 interaction evidence(s), but TF has a validated binding site
- / + / -	Less than 1 interaction, but TF has a predicted binding site

Table 4.2 Summary table showing potential transcription factor binding sites on the target gene promoters. The online TRANSFAC database was used to identify potential transcription factor (TF) binding sites in the target promoter sequence. Match analysis was performed, which uses positional weight matrices (preferred nucleotide patterns) determined from TRANSFAC's broad compilation of experimental and theoretical determined binding sites to search a DNA sequence for potential TF binding sites. This table shows the results retrieved after TRANSFAC analysis, where the promoter sequences were analysed for the following genes; PRL, NR3C1, PGR, HSD11B2, HSD11B1, AR, WT1, FKBP5 and FKBP4. Additionally ESR1, STAT4 and STAT5A were included in the analysis out of interest due to their involvement within the human endometrium. These promoter sequences were analysed for potential binding sites for the following TFs; GR, AR, WT1 and PGR. This table demonstrates whether identified potential binding sites have been validate or are predicted and whether there is any evidence of an interaction of the TF with the respective gene which is indicated by the legend.

A selection of potential GR binding sites were identified in target promoters and primers were designed. Chromatin immunoprecipitation was subsequently performed to elucidate whether GR was directly binding to the promoter region of these genes and therefore determine if GR can modulate the transcription of these genes.

			Promoter Position		
Gene Promoter	**Transcription Factor**	**Sequence**	**From (-bp)**	**To (-bp)**	**Similarity Sequence Score**
NR3C1	Glucocorticoid receptor binding site	taaGCACatattgtaagta	-3358	-3340	0.851
	Progesterone receptor binding site	aggtcaagcagTGTTctca	-2401	-2383	0.89
		atggggagatgTGTTctat	-2713	-2695	0.887
	Androgen receptor binding site	tgtaagcctgttGTCCtca	-3214	-3196	0.904
AR	Glucocorticoid receptor binding site	agaGTACatgctgtatagt	-3921	-3913	0.873
		ctgGTACaaacagtgaact	-3250	-3232	0.835
	Progesterone receptor binding site	taattgtctctTGTTctta	-3214	-3196	0.845
		gtttacaagttTGTTctat	-3013	-2995	0.844
	Androgen receptor binding site	ttgtgtctatgtGTCCtct	-2781	-2763	0.906
		tcaggcctttgtGTTCtgg	-2460	-2442	0.946
	Androgen receptor binding site	tgtagacacataGTTCtcc	-2707	-2689	0.879
	Repressive binding sites for glucocorticoid receptor	ggcctccaGGAAatc	-1295	-1281	0.897
PGR	Repressive binding sites for glucocorticoid receptor	tatatccaGGAGaaa	-2786	-2772	0.894
	Glucocorticoid receptor binding site	ttaGCACagctagaccttg	-1898	-1880	0.842
		gcaGGACatgttggtcgca	-1427	-1409	0.839

Table 4.3 Table showing potential transcription factor binding sites for GR in the promoter region of the sex steroid hormone receptor genes; NR3C1, AR and PGR. The online TRANSFAC database was used to identify potential GR binding sites in the target promoter sequences. Match analysis was performed, which uses positional weight matrices (preferred nucleotide patterns) determined from TRANSFAC's broad compilation of experimental and theoretical determined binding sites to search a DNA sequence for potential GR binding sites. This table shows the results retrieved after TRANFAC analysis, where the NR3C1, AR and PGR promoters had potential binding sites for GR (either GREs or negative GREs). This table demonstrates the promoter region under investigation, the transcription factor binding site, the binding site sequence, the position of the potential binding site within the promoter sequence and the sequence similarity score between the input promoter sequence and the known sequence of TF binding sites.

			Promoter Position		
Gene Promoter	**Transcription Factor**	**Sequence**	**From (-bp)**	**To (-bp)**	**Sequence Similarity Score**
PRL	Glucocorticoid modulatory element binding protein 2	tACGTgacagaagaa	-3026	-3012	0.787
	Glucocorticoid receptor	aatacaaacttTGTTctaa	-2968	-2950	0.89
		gaagtaataatTGTTcctt	-1413	-1395	0.892
		cacacatttcaTGTTctag	-550	-532	0.881
FKBP4	Mineralcorticoid receptor response element	gagaaacagtaaGTTCctg	-3300	-3282	0.839
	Glucocorticoid receptor	ttgagaccagaTGTTcgag	-2211	-2193	0.881
	Androgen receptor binding site	gcagttcactctGTACtgc	-610	-592	0.92
	Progesterone receptor binding site	taaggaaaattTGTTctct	-161	-143	0.926
	Repressive binding sites for glucocorticoid receptor	ggtctcccGGAGttt	-2634	-2620	0.865
		gcCGCCggggagatc	-1225	-1211	0.883
FKBP5	Repressive binding sites for glucocorticoid receptor	gggcaccaGGAGacg	-3927	-3913	0.882
		agacgccaGGAGaca	-1332	-1318	0.875
	Repressive binding sites for glucocorticoid receptor	ccCTCCgaggaggaa	-1131	-1117	0.88
	Androgen receptor binding site	ctggtgctttgTATTctct	-3432	-3414	0.812
	Androgen receptor binding site	agtgtactcactGTGCttg	-2301	-2283	0.952
	Mineralcorticoid receptor response element	ccatcacagagaGTACaca	-2328	-2310	0.82
WT1	Repressive binding sites for glucocorticoid receptor	tggctccaGGAGtta	-1948	-1934	0.877
	Repressive binding sites for glucocorticoid receptor	ctCTCCagtgagacg	-1301	-1287	0.866
	Glucocorticoid receptor	agggacttatatGTCCtag	-846	-828	0.872
	Glucocorticoid receptor	gctGAACacctagtacaat	-602	-584	0.871
	Androgen receptor binding site	aactctctttctGTTCtgg	-473	-455	0.959
HSD11B1	Progesterone receptor binding site	gtggcatctctTGTTcttc	-3277	-3259	0.916
		taggtacttatTGTTctgg	-2700	-2682	0.979
	Androgen receptor binding site	atagaacgtatgGTTCtct	-2222	-2204	0.899
	Repressive binding sites for glucocorticoid receptor	ctcctctgGGAAaga	-837	-859	0.811
HSD11B2	Repressive binding sites for glucocorticoid receptor	gggctcctGGAGtct	-3952	-3938	0.882
		ggggtcatGGAGagg	-576	-562	0.83
	Repressive binding sites for glucocorticoid receptor	caCTCCaaggggaag	-2904	-2890	0.884
		gcCTCCcctgagatt	-2340	-2326	0.87
	Glucocorticoid receptor	gaaGGACagagagttacca	-2667	-2649	0.896
		cagGCACcaaggtcatgt	-2526	-2508	0.862
	Glucocorticoid receptor	ccggttgtgcgtGTCCtca	-1387	-1369	0.872
	Androgen receptor binding site	agcggtcgtcctGTTCccc	-1642	-1624	0.911
	Androgen receptor binding site	cacacacactctGTCCttc	-444	-426	0.927

Table 4.4 Table showing potential transcription factor binding sites for GR in the promoter region of potential GR target genes. The online TRANSFAC database was used to identify potential GR binding sites in the target promoter sequences of the following genes: PRL, FKBP4, FKBP5, WT1, 11BHSD1 and 1BHSD2. Match analysis was performed, which uses positional weight matrices (preferred nucleotide patterns) determined from TRANSFAC's broad compilation of experimental and theoretical determined binding sites to search a DNA sequence for potential GR binding sites. This table shows the results retrieved after TRANFAC analysis, where all target promoters under investigation had potential binding sites for GR (either GREs or negative GREs). This table demonstrates the promoter region under investigation, the transcription factor binding site, the binding site sequence, the position of the potential binding site within the promoter sequence and the sequence similarity score between the input promoter sequence and the known sequence of TF binding sites.

4.2.6.3 Occupancy of GRE binding sites within the promoter region of target genes by GR during the decidual transformation of endometrial stromal cells

Chromatin immunoprecipitation (ChIP) was used to determine site specific GR occupancy of the promoter of the following genes; NR3C1, PRL, WT1, FKBP5, PGR, AR, HSD11B1 and HSD11B2 following 48h treatment of the HESC cell line with either cAMP, cAMP + F, cAMP + E, E, F or RU486 and compared to untreated cells.

It was found that treatment of the HESC cell line with the decidualization stimulus in the presence or absence of E and F, E alone, F alone or the GR antagonist; RU486 did not result in any significant increase in the GR recruitment to the promoter of NR3C1 (-3214/-3196bp) compared to either the control untreated cells or the cells which had undergone ChIP with a negative IgG control antibody which is corresponding to the host species in which the antibody of interest was raised i.e. anti-rabbit IgG (figure 4.25). This control IgG antibody was run in parallel to the anti-GR target antibody.

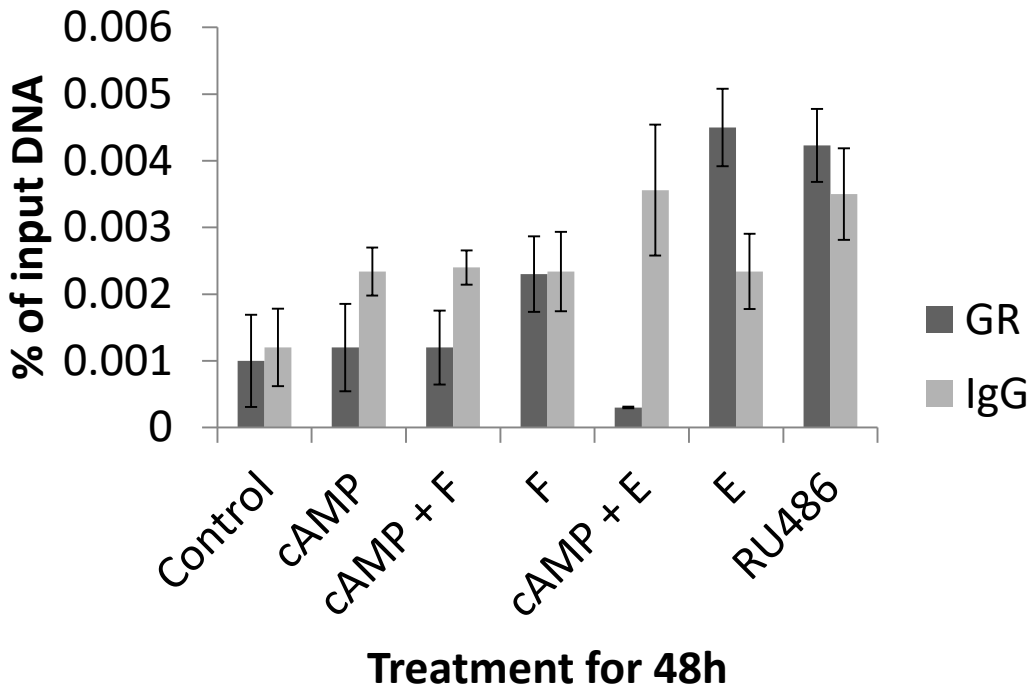


Figure 4.25 The investigation of the potential interaction of GR with the NR3C1 promoter between the regions of -3214/-3196bp. Chromatin immunoprecipitation (ChIP) was performed by the immunoprecipitation of the extracted HESC chromatin with anti-GR and consequent qPCR analysis of the DNA using specific primers for the target binding site on the NR3C1 promoter. This allowed for the determination of GR enrichment at one potential GR binding site on the NR3C1 promoter (-3214/-3196bp) after the cells had been treated for 48h with either cAMP only, cAMP + F, F only, cAMP + E, E only or the GR antagonist; RU486. Normal rabbit immunoglobulin (IgG) was used as the immunoprecipitation (IP) control. Statistical analysis was performed using the ANOVA statistical test followed by the Student's t test. Values are averaged and bars indicate ± SD.

Interestingly, treatment of the HESC cell line with cAMP only, cAMP + F and cAMP + E resulted in the significant enrichment of GR on its potential binding site on the dPRL promoter (-1413/-1395bp) compared to untreated control cells ($p \leq 0.05$) (figure 4.26). This indicates that the direct interaction of GR with the dPRL promoter may be altering the transcription of the dPRL gene. The highest percentage of enrichment was observed when the HESC cell line was either treated with the in vitro decidualization stimulus only or cAMP + E. Treatment of the HESC cell line with either F only, E only or the RU486 antagonist did not result in any significant changes in the enrichment of GR on the promoter of dPRL (figure 4.26). It is also important to note that under unstimulated conditions, GR seems to be present and enriched its specific binding site on the dPRL promoter, which suggests that GR may regulate basal dPRL expression within the endometrial stromal cells (figure 4.26).

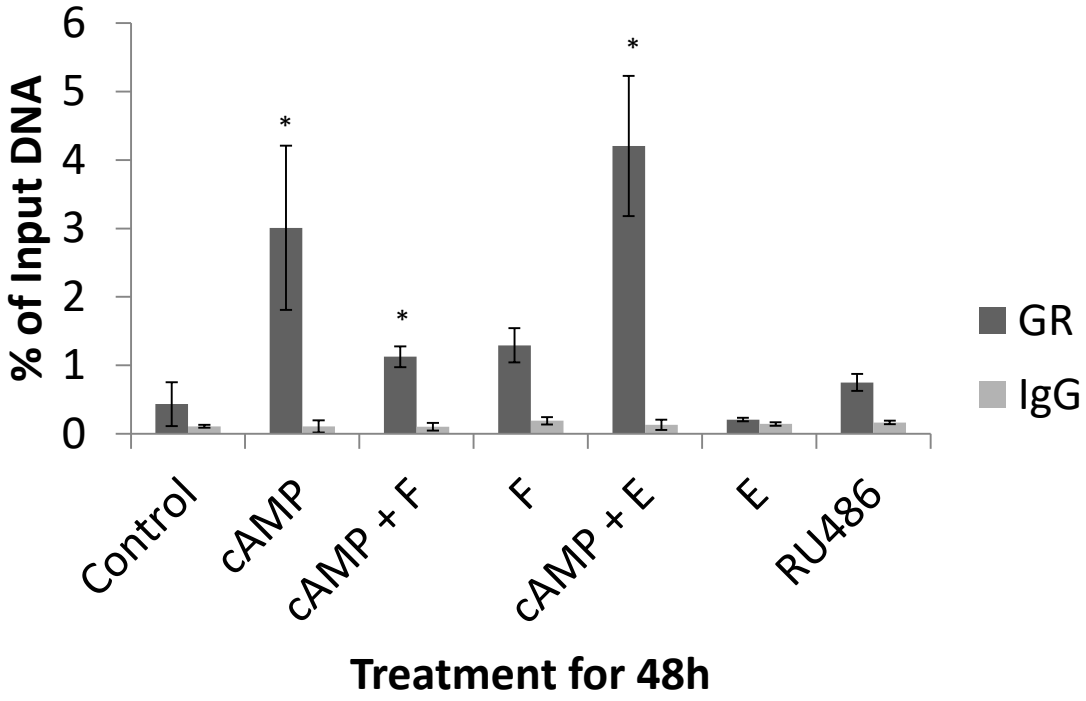


Figure 4.26 The investigation of the potential interaction of GR with the dPRL promoter between the regions of -1413/-1395bp. Chromatin immunoprecipitation (ChIP) was performed by the immunoprecipitation of the extracted HESC chromatin with anti-GR and consequent qPCR analysis of the DNA using specific primers for the target binding site on the dPRL promoter. This allowed for the determination of GR enrichment at one potential GR binding site on the dPRL promoter (-1413/-1395bp) after the cells had been treated for 48h with either cAMP only, cAMP + F, F only, cAMP + E, E only or the GR antagonist; RU486. Normal rabbit immunoglobulin (IgG) was used as the immunoprecipitation (IP) control. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ are considered significant.

Furthermore, the enrichment of GR on the promoter region of WT1 was also investigated. It was found that treatment of the HESC cell line with cAMP + F resulted in a significant enrichment of GR on its potential binding site on the WT1 promoter (-846/-828bp) compared to the untreated control cells ($p \leq 0.05$) (figure 4.27). Treatment with cAMP + E also seemed to result in an enrichment of GR on the WT1 promoter, but this was deemed to be not significant. However, treatment with E only resulted in a significant reduction of the enrichment of GR at the WT1 promoter compared to the control ($p \leq 0.05$). Treatment with cAMP, F, RU486 or cAMP + E co-treatment did not result in any significant differences in GR enrichment at the specific binding site on the promoter region of WT1 (figure 4.27). Additionally, similar to the dPRL results obtained for unstimulated conditions, GR seems to be present and enriched its specific binding site on the WT1 promoter also, which suggests that GR may regulate basal WT1 expression within the endometrial stromal cells (figure 4.27).

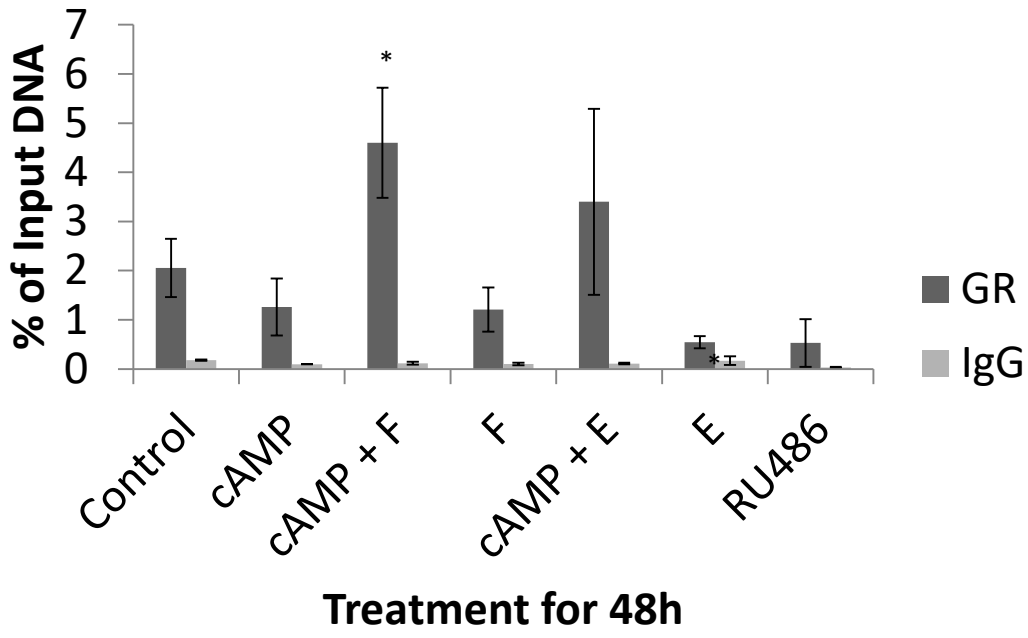


Figure 4.27 The investigation of the potential interaction of GR with the WT1 promoter between the regions of -846/-828bp. Chromatin immunoprecipitation (ChIP) was performed by the immunoprecipitation of the extracted HESC chromatin with anti-GR and consequent qPCR analysis of the DNA using specific primers for the target binding site on the WT1 promoter. This allowed for the determination of GR enrichment at one potential GR binding site on the WT1 promoter (-846/-828bp) after the cells had been treated for 48h with either cAMP only, cAMP + F, F only, cAMP + E, E only or the GR antagonist; RU486. Normal rabbit immunoglobulin (IgG) was used as the immunoprecipitation (IP) control. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ are considered significant.

qPCR post ChIP analysis was also carried out to determine the possibility of GR enrichment on the promoter region of FKBP5 at a specific GR binding site (-2328/-2310bp). It was found that treatment of the HESC cell line with cAMP + E resulted in a significant enrichment of GR on its potential binding site on the FKBP5 promoter region (-2328/-2310bp) compared to the untreated control cells ($p \leq 0.05$) (figure 4.28). Treatment with cAMP, cAMP + F, F, E or RU486 did not result in any significant alteration of enrichment of GR on the FKBP5 promoter at the specific binding site under investigation (figure 4.28).

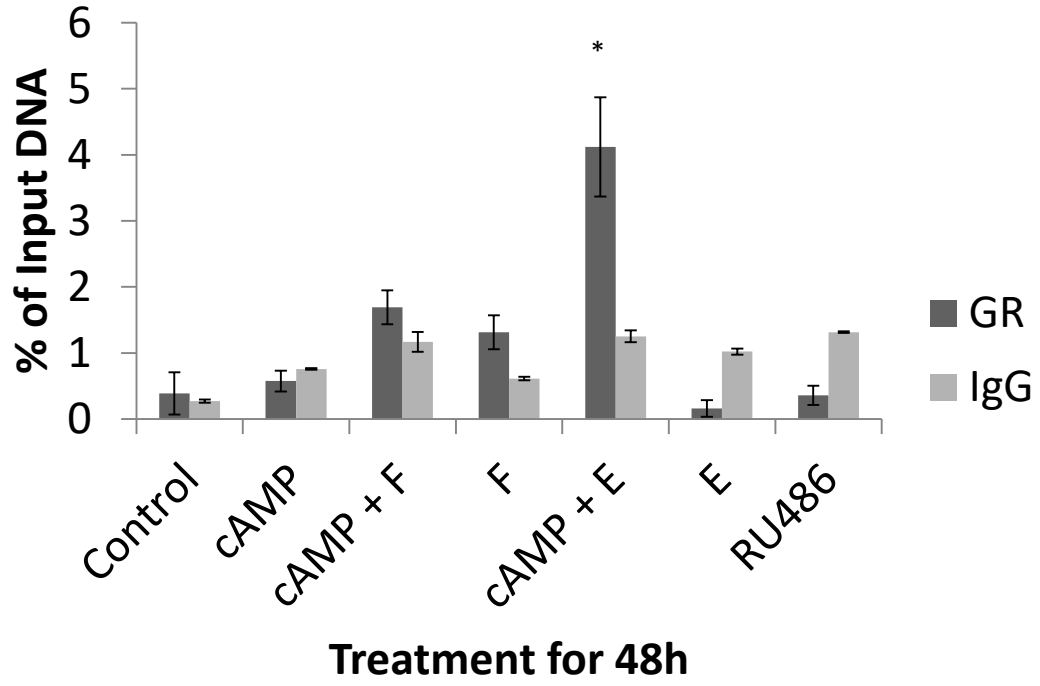


Figure 4.28 The investigation of the potential interaction of GR with the FKBP5 promoter between the regions of -2328/-2310bp. Chromatin immunoprecipitation (ChIP) was performed by the immunoprecipitation of the extracted HESC chromatin with anti-GR and consequent qPCR analysis of the DNA using specific primers for the target binding site on the FKBP5 promoter. This allowed for the determination of GR enrichment at one potential GR binding site on the WT1 promoter (-2328/-2310bp) after the cells had been treated for 48h with either cAMP only, cAMP + F, F only, cAMP + E, E only or the GR antagonist; RU486. Normal rabbit immunoglobulin (IgG) was used as the immunoprecipitation (IP) control. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ are considered significant.

Furthermore, the enrichment of GR on the promoter region of the steroid hormone receptor; PGR was also investigated. Although differences in the percentage of input DNA bound to the promoter region of PGR was observed, it was found overall that treatment of the HESC cell line with cAMP, F, E or RU486 did not result in any significant differences of the enrichment of GR on its potential binding site on the PGR promoter (-1898/-1880bp) (figure 4.29). However, cells that were treated with cAMP + F resulted in a significant increase in the levels of GR enrichment at the promoter region of PGR compared to the control cells

(p≤0.05). Additionally, cells treated with cAMP + E, also indicated a significant increase in the levels of GR enrichment at the PGR promoter compared to untreated control cells (p≤0.05) (figure 4.29). Under unstimulated conditions, GR seems to be present and enriched its specific binding site on the PGR promoter, which suggests that GR may regulate basal PGR expression within the endometrial stromal cells (figure 4.29).

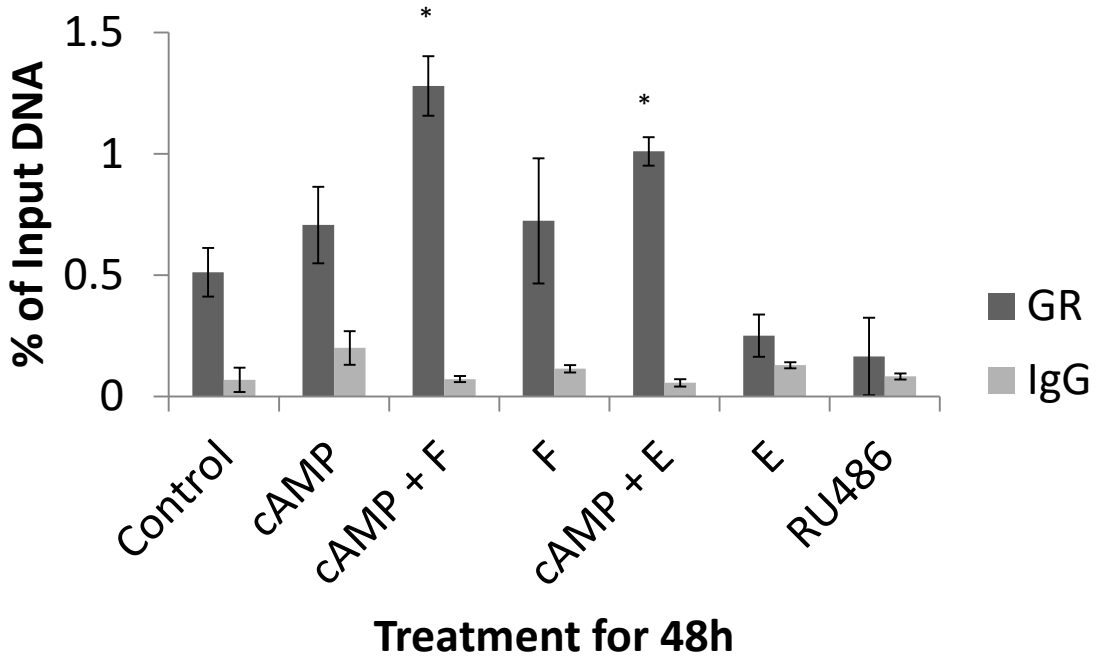


Figure 4.29 The investigation of the potential interaction of GR with the PGR promoter between the regions of -1898/-1880bp. Chromatin immunoprecipitation (ChIP) was performed by the immunoprecipitation of the extracted HESC chromatin with anti-GR and consequent qPCR analysis of the DNA using specific primers for the target binding site on the PGR promoter. This allowed for the determination of GR enrichment at one potential GR binding site on the PGR promoter (-1898/-1880bp) after the cells had been treated for 48h with either cAMP only, cAMP + F, F only, cAMP + E, E only or the GR antagonist; RU486. Normal rabbit immunoglobulin (IgG) was used as the immunoprecipitation (IP) control. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

qPCR post ChIP analysis was also carried out to determine the possibility of GR enrichment on the promoter region of AR at a specific GR binding site (-3013/-2995bp). Interestingly, it was found that only treatment of the HESC cell line with the GR antagonist; RU486 or those co-treated with cAMP + E, resulted in an enrichment of GR at its potential binding site on the AR promoter region compared to the untreated control cells (p≤0.05) (figure 4.30). However, the 48h treatment of the HESC cell line with cAMP, cAMP + F, F, or E did result in variations in the levels of GR enrichment at its specific binding site located within the AR promoter, no significant differences in GR enrichment were found (figure 4.30). However,

these results have to be further investigated due to high values of enrichment being obtained with the IgG control for both the cells treated with E or with RU486 (figure 4.30). Similar to that seen in previous analysis, under unstimulated conditions, GR seems to be present and enriched its specific binding site on the AR promoter, which suggests that GR may regulate basal AR expression within the endometrial stromal cells (figure 4.30).

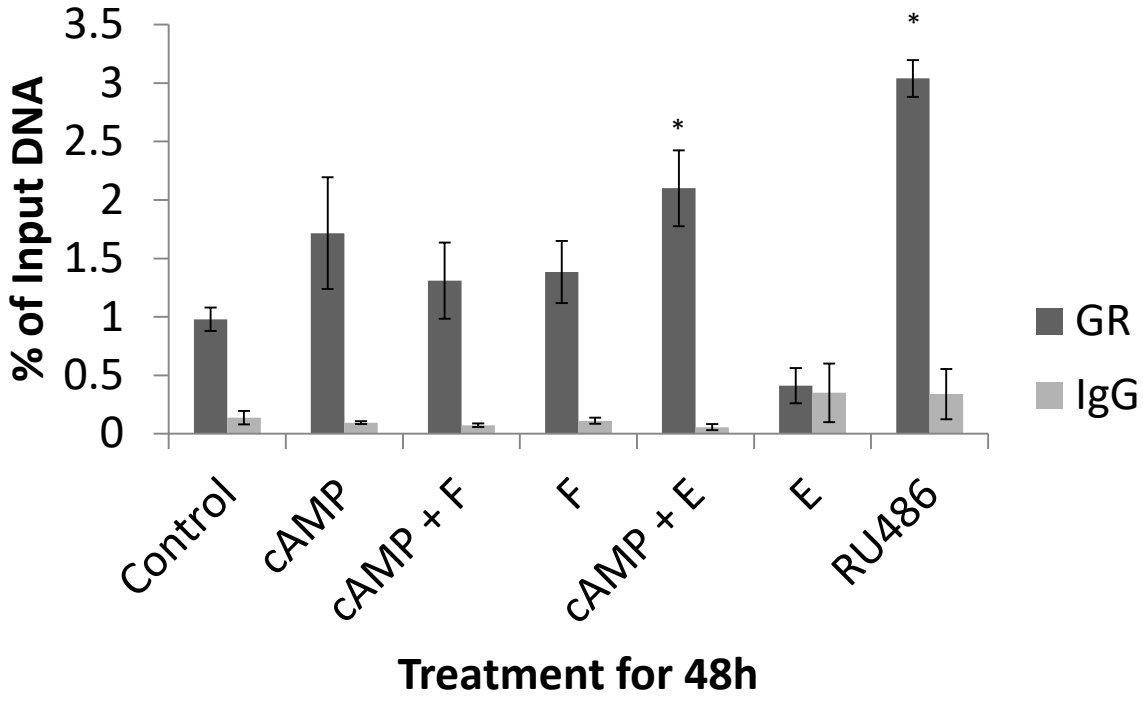


Figure 4.30 The investigation of the potential interaction of GR with the AR promoter between the regions of -3013/-2995bp. Chromatin immunoprecipitation (ChIP) was performed by the immunoprecipitation of the extracted HESC chromatin with anti-GR and consequent qPCR analysis of the DNA using specific primers for the target binding site on the AR promoter. This allowed for the determination of GR enrichment at one potential GR binding site on the AR promoter (-3013/-2995bp) after the cells had been treated for 48h with either cAMP only, cAMP + F, F only, cAMP + E, E only or the GR antagonist; RU486. Normal rabbit immunoglobulin (IgG) was used as the immunoprecipitation (IP) control. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

Finally, the enrichment of GR was also investigated after specific treatments at the promoter region of the 11β-HSD enzymes, where both 11BHSD1 and 11BHSD2 were analysed. ChIP and qPCR analysis revealed that in the HESC cell line, after 48h treatment with both E and RU486, that there was a significant reduction in the enrichment of GR on the promoter region of 11BHSD1 at a binding site under investigation (-2700/-2682bp) compared to the untreated control cells (p≤0.05) (figure 4.31). Treatment with cAMP + E did appear to result in an increase in the levels of GR enrichment at the 11BHSD1 promoter but this was not deemed to be statistically significant. Additionally, treatment of the HESC cell line with

cAMP, cAMP + F, F and RU486 did not result in any significant differences in the enrichment of GR on the promoter region of 11BHSD1 (figure 4.31).

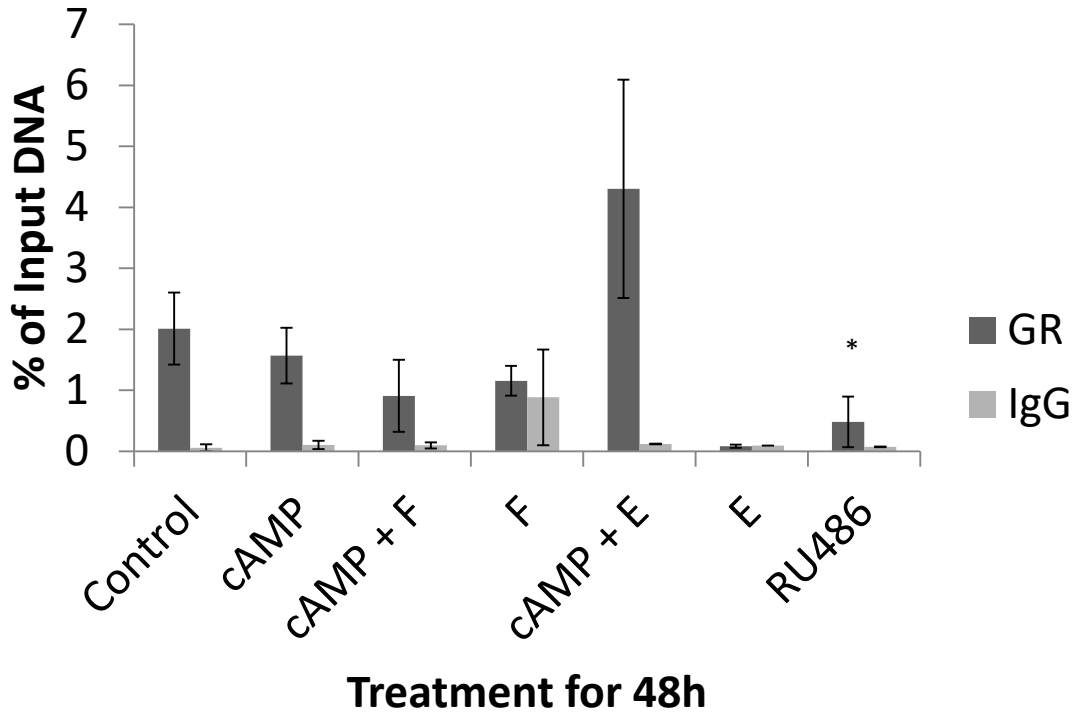


Figure 4.31 The investigation of the potential interaction of GR with the 11BHSD1 promoter between the regions of -2700/-2682bp. Chromatin immunoprecipitation (ChIP) was performed by the immunoprecipitation of the extracted HESC chromatin with anti-GR and consequent qPCR analysis of the DNA using specific primers for the target binding site on the 11BHSD1 promoter. This allowed for the determination of GR enrichment at one potential GR binding site on the 11BHSD1 promoter (-2700/-2682bp) after the cells had been treated for 48h with either cAMP only, cAMP + F, F only, cAMP + E, E only or the GR antagonist; RU486. Normal rabbit immunoglobulin (IgG) was used as the immunoprecipitation (IP) control. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

Similar results were obtained for the 11BHSD2 promoter, where treatment with the HESC cell line with cAMP + E also resulted in a significant increase in the enrichment of GR at the potential GR binding site within the promoter region of 11BHSD2 (-2667/-2649bp) (figure 4.32). However, 48h treatment with cAMP, cAMP + F, F, E and RU486 did not result in any significant differences in GR enrichment at the specific binding site under investigation located within the promoter region of 11BHSD2 (figure 4.32).

Under unstimulated conditions, GR seems to be present and enriched its specific binding site on both the 11BHSD1 and 1BHSD2 promoters, which suggests that GR may regulate the basal expression levels of these enzymes within the endometrial stromal cells (figure 4.32).

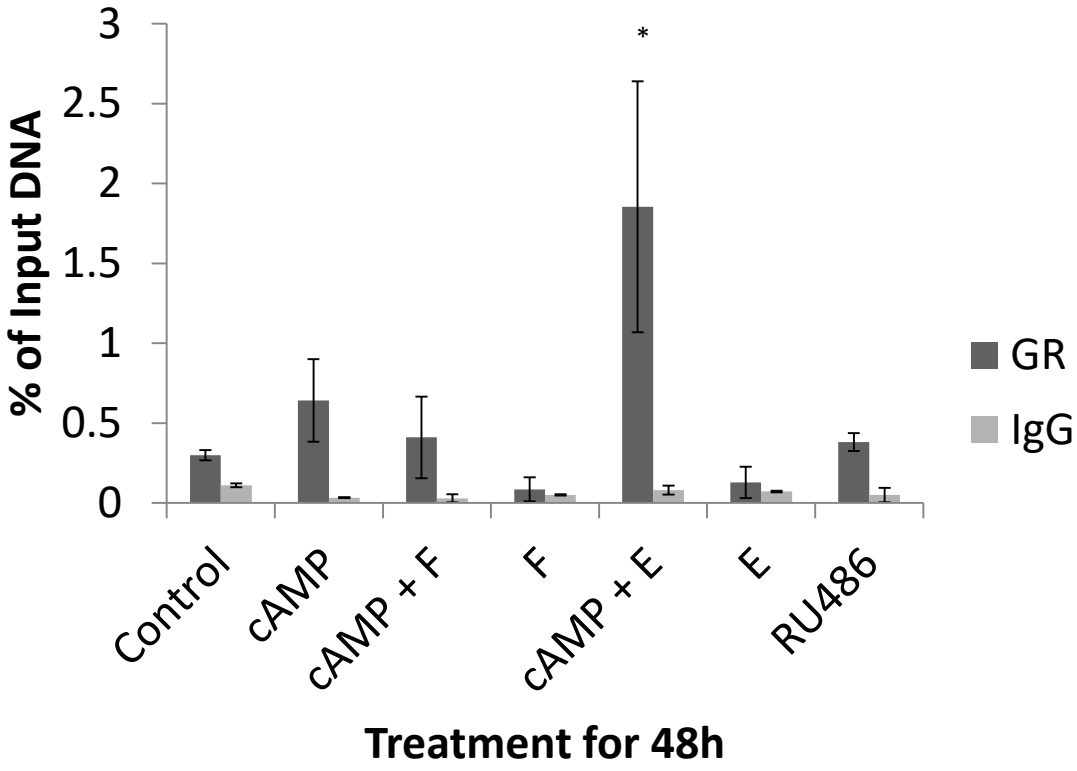


Figure 4.32 The investigation of the potential interaction of GR with the 11BHSD2 promoter between the regions of -2667/-2649bp. Chromatin immunoprecipitation (ChIP) was performed by the immunoprecipitation of the extracted HESC chromatin with anti-GR and consequent qPCR analysis of the DNA using specific primers for the target binding site on the 11BHSD2 promoter. This allowed for the determination of GR enrichment at one potential GR binding site on the 11BHSD2 promoter (-2667/-2649bp) after the cells had been treated for 48h with either cAMP only, cAMP + F, F only, cAMP + E, E only or the GR antagonist; RU486. Normal rabbit immunoglobulin (IgG) was used as the immunoprecipitation (IP) control. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

It is important to note that ChIP analyses the native state of transcription factor binding, therefore occupancy of adjacent binding sites or indeed dynamic chromatin structure will influence the observed GR occupancy within different cellular contexts. Hence, in these experiments, binding and the enrichment of GR at the promoter regions under investigation may be influenced by the binding of other transcription factors and vice versa.

Even though interestingly results were obtained for the ChIP and qPCR analysis, it is crucial that these experiments are repeated with both the HESC cell line and also with primary ESCs isolated from fertile patients. Additionally, TRANSFAC analysis identified numerous potential GR binding sites on the promoter regions of target genes, therefore more research is needed to investigate these binding sites in addition to those identified and explored in this thesis.

4.3 Discussion

This chapter explores the expression of GR within the endometrial stromal compartment and the effect of the GR signalling pathway in the presence and absence of the stress hormones during the crucial process of decidualization, in otherwise fertile endometrium. Therefore, this chapter aimed to elucidate whether stress exposure and the GR signalling pathway had any effect on crucial processes and expression of key proteins within the endometrium which may impact the success of implantation.

This was achieved by determining the effect of both cortisol and cortisone exposure on ESCs isolated from fertile patients in addition to using the well-established 'HESC' cell line, which proved to be an overall good in vitro model of the fertile endometrium to assess the GR signalling pathway during decidual transformation. Targets of the GR signalling pathway were also elucidated and identified in this study. Work presented in this chapter provides us with novel molecular evidence to support the hypothesis that excess stress levels may have a detrimental impact on fertility status, particularly within females.

Data reported in this chapter identifies GR to be expressed in the stromal compartment of the fertile endometrium, hence confirming the IHC data previously reported in chapter 3 (figure 4.1,4.2 and figure 3.3 respectively). Furthermore, the 11β-HSD enzymes are also expressed within the stromal compartment which is also in agreement with results presented in chapter 3 (figure 4.1, 4.2 and figure 3.9 respectively). The HESC cell line expresses the GR isoforms (α and β) and the 11β-HSD type 1 and type 2 isoforms at similar levels as the primary ESCs isolated from fertile patients (figure 4.1 and 4.2), and therefore provides a good model for further chromatin immunoprecipitation (ChIP) experiments. These results are in agreement with previous studies which also demonstrated that 11β-HSD1 and GR are expressed within the human endometrial stromal compartment (McDonald et al, 2006; Kuroda et al, 2013; Bamberger et al, 2001). Furthermore, McDonald and colleagues identified that the 11β-HSD2 enzyme is expressed at lower levels in the endometrial stroma compared to the glandular region and is also expressed at lower levels in the stromal compartment compared to 11β-HSD1 (McDonald et al, 2006), which is in accordance with results presented in this chapter.

It was observed that GRα and GRβ protein expression levels did not change in ESCs stimulated in vitro to decidualize (figure 4.6), whereas reductions in GR expression at the mRNA level were observed (figure 4.7). The mRNA data is in accordance with the ex vivo data presented in chapter 3, which also shows reductions in the endometrial GR expression during the secretory phase of the menstrual cycle (figure 3.3). Additionally, the findings

presented in figure 4.7 are concurrent with previous findings that suggest that GR expression is inhibited slightly during the decidual response (Kuroda et al, 2013).

The principal discovery in this chapter was that the treatment of ESCs with the active stress hormone; cortisol, significantly inhibited the decidualization process, in terms of alterations of ESC morphology using quantitative morphological analysis of cell monolayers post-treatment (figure 4.3) and reductions in the expression levels of key decidual markers (dPRL and IGFBP-1) (figure 4.4 and 4.5). As previously mentioned, decidual transformation of the ESCs resulted in a decrease in the endometrial expression of the primary GR isoforms; GRα and GRβ and 11β-HSD1 within the stromal compartment (figure 4.7). This effect was further enhanced during decidualization in the presence of cortisol exposure during decidual transformation of the fertile endometrium resulting in a further decrease in the endometrial protein expression of the primary GR isoforms; GRα and GRβ and 11β-HSD1 within the stromal compartment (figure 4.6). These results indicate that cortisol exposure results in deficiencies of the decidualization process, which may have a significant impact on successful implantation and pregnancy maintenance due to the ESCs reduced capacity to evolve into their characteristic decidual cell phenotype and express the genes and proteins critical to maintain this process (Gonzalez et al, 2012; Telgmann and Gellersen, 1998). Overall these effects suggest cortisol may be an inhibitor of the decidual response. This seems to be in contradiction with the notion that GCs are administered systemically to patients undergoing ARTs, such as IVF and ICSI, with the aim to improve the success rates of implantation and ultimately pregnancy (Kalampokas et al, 2017). However, it must be noted that the GC under investigation in this study is the active stress hormone cortisol, which is naturally produced and circulated within the body daily. Whereas the GCs used during ARTs are corticosteroids such as dexamethasone which are synthetic and not present within the body unless exogenously administered.

It is known that the abundance of active GCs (such as cortisol) within tissues (including the endometrium) is dependent on the expression levels of the 11βHSD enzymes, where the type 1 isoform catalyses the conversion of the inactive ligand; cortisone, to cortisol and the type 2 isoform catalyses the reverse reaction (Kuroda et al, 2013). Our results show that upon successful decidualization, ESCs have a significantly increased expression of 11β-HSD1 at the mRNA level (figure 4.7). This finding supports several previous reports which hypothesise 11β-HSD1 is having a role within endometrial decidualization, due to its up-regulation during this critical process (Chan et al, 2007; Newton, 2000; Kuroda et al, 2013). The up-regulation of 11β-HSD1 is maintained at the mRNA level with the addition of cortisol during decidualization of ESCs, which is yet to be reported on (figure 4.7). Furthermore, qPCR analysis revealed that in vitro decidualization results in a significant

decrease in the expression of 11β-HSD2 isoform (figure 4.7). This, in part, supports previous research which suggests that endometrial 11β-HSD2 expression is absent during decidualization (McDonald et al, 2006) and differs to 11β-HSD1 expression as it is not up-regulated upon differentiation of ESCs into decidual cells in vitro (Takano, 2007). It is known that an optimal concentration of cortisol is needed for the homeostasis of the endometrium, but dysregulation of cortisol bio-availability due to over-expression of the 11β-HSD1 may have detrimental effects (Zhu et al, 2016). Therefore the expression of 11β-HSD1 in infertile pathologies needs to be investigated.

These results support previous research which suggests that along with the well-established sex steroid hormone signalling pathways, the GR signalling pathway is also having a significant role within the human endometrium, especially during the decidual response (Whirledge and Cidlowski, 2013). This study supports GRα knock-out (KO) mice studies, which indicate that the GR signalling pathway has a significant role in implantation within the mouse endometrium (Whirledge et al, 2015). Future work to establish the effect of GRα knock-out in ESCs would elucidate whether GR is having a direct effect on the decidual process.

It is known the primary binding receptor for cortisol is GR; however, cortisol also has a high affinity for the mineralocorticoid receptor (MR) due to its structural and functional similarities with GR. Moreover, 11β-HSD1 is normally found co-localised with GR, whereas 11β-HSD2 is commonly found in tissues expressing MR (Yang et al, 1997; Engeli et al, 2004). MR is known to be expressed within the human endometrium within both the stromal and glandular compartments and displays an up-regulation of endometrial expression during the secretory phase of the menstrual cycle (McDonald et al, 2006). In accordance with previous results, work presented in this chapter demonstrates an up-regulation of MR expression with concomitant down-regulation of GR in decidual HESC cells (figure 4.8). This has been previously reported by Kuroda and colleagues, who described that progesterone activation of ESCs leads to the transcriptional regulation of distinct GR and MR gene networks involved in epigenetic programming and retinoid metabolism respectively (Kuroda et al, 2013). These authors performed a global analysis of the epigenetic status in addition to micro-array analysis to identify general pathways and genes modulated by GR and MR. However, Kuroda and colleagues incubated the ESCs with the inert GC; cortisone to stimulate its conversion to the active ligand cortisol, through 11β-HSD1 activity, given that the enzyme levels were found to be elevated in the decidual cells (Kuroda et al, 2013). Even though cortisone was included in the current study, data presented in this chapter obtained after stimulation with cortisol was deemed to be of increased clinical relevance. The use of cortisol also allowed for the management of cortisol

treatment concentrations independently of enzymatic activity and allowed for the clarification of the effect of GR activation via its active ligand on the decidual response within the endometrium and furthermore its effect on master regulators of decidualization.

Master regulators of decidual transformation included in this study included Wilms' tumour protein 1 (WT1), FK506 binding protein 5 (FKBP5) and FK506 binding protein 4 (FKBP4).

It is known that WT1 is expressed in the endometrium during the 'window of implantation' in the fertile patients and may be a master regulator of the decidual response. Results in this chapter support previous work conducted by the RBGO group that in vitro decidualization of ESCs results in an increase in WT1 expression (figure 4.10) (Gonzalez et al, 2012). Interestingly, it appears that the presence of cortisol during decidual transformation may also be having a significant effect on endometrial WT1 levels. This supports the notion that cortisol and hence the GR signalling pathway are having a direct effect on decidual transformation of ESCs. It is important to note that the biology of WT1 is complex, which is reflected by the fact that at least 24 alternative isoforms exist due to post-translational alterations (Hohenstein and Hastie, 2006). Therefore more work is crucial to identify the effect of cortisol exposure during decidual transformation on the multiple WT1 isoforms. Additionally, there is much data supporting the involvement of WT1 within malignant cells, which suggests a potential role for WT1 as an oncogene. This is supported by studies which have discovered over-expression of WT1 various malignancies (Koesters et al, 2004; Loeb et al 2001; Nakatsuka et al, 2006), including endometrial carcinomas (Coosemans et al, 2008). This is of interest, as treatment of the ESCs with F alone resulted in increases in the endometrial expression of WT1 (figure 4.9) which may be of interest in the field of endometrial oncology.

In addition to WT1, the levels of FKBP5 (protein and mRNA levels) and FKBP4 (mRNA levels only) were investigated. FKBP5 and FKBP4 are members of the immunophilin protein family and are involved in immune-regulation, the regulation of basic protein folding and trafficking and more importantly they play a key role in the regulation of the steroid hormone receptors as part of the heat shock protein 90 (HSP90) steroid receptor complex (Bali et al, 2016; Davies and Sánchez). Both immunophillins are able to act as co-chaperones with HSP90 and interact with the ligand binding domain of PR (Yang et al, 2012) and GR (Bali et al, 2016). Previous research has demonstrated the importance of FKBP immunophillin expression in regulating decidualization within the endometrium (Yang et al, 2012; Schatz et al, 2015). Interestingly, our novel findings revealed that cortisol exposure during decidual transformation results in the induction of the endometrial expression of FKBP5 (figure 4.10). It has been found that the over-expression of FKBP5 results in reductions in hormone binding affinity and nuclear translocation of GRα (Patel et

al, 2016). In addition to GR, it is also known that FKBP5 is able to attenuate hormone responses of PR (Yang et al, 2012); therefore increases in the expression of FKBP5 may result in the reduction of the decidual response in the fertile endometrium. Furthermore, cortisol exposure during decidual transformation results in the reduction of FKBP4 expression (figure 4.10). This supports the morphological data and decidual marker data presented in this chapter, which suggests that cortisol exposure in the presence of cAMP results in the inhibition of the decidual response in vitro. It has been previously reported that FKBP4 is crucial in regulating endometrial decidualization and knock-down of FKBP4 results in the inhibition of decidualization in terms of cell morphology and the expression of key decidual markers (Yang et al, 2012). Therefore reductions in FKBP4 expression during decidualization due to F exposure may be contributing to the inhibition of the decidual response.

In addition to cortisol, it was also decided to investigate the effect of the inert GC; cortisone on decidual transformation of ESCs. This was due to the observation that during in vitro decidual transformation, ESCs profoundly up-regulate 11β-HSD1, the enzyme responsible for converting the inert cortisone to the active ligand cortisol. As seen with cortisol exposure, the presence of cortisone during decidual transformation also resulted in the inhibition of the decidual response in terms of ESC morphology (figure 4.11). However, no significant reductions in the expression of the key decidual markers were observed (figure 4.12 and 4.13). This indicates that the inert GC does not inhibit the decidual response to the same extent as the active ligand; cortisol, and resulted in a less pronounced effect on the expression of the master regulators of decidualization (figure 4.17). This could be potentially due to the rate of conversion of cortisone to cortisol after 48h, which may not result in the same amounts of cortisol used in the direct stimulation of samples.

Cortisone also resulted in the decrease in the endometrial expression of 11β-HSD2 at the mRNA level during decidual transformation, whereas a significant induction in 11β-HSD1 expression was observed (figure 4.15). Therefore the ESCs are favouring a local environment with increased catalytic conversion of cortisone to cortisol by up-regulating 11β-HSD1 and down-regulating the potent de-activator of GCs, which prevents the active ligand being converted to the inert GC (Smith et al, 1997). This suggests that the expression of the 11β-HSD isoforms within the endometrium during decidual transformation is significantly affected by the local bio-availability of the GCs; cortisol and cortisone. Alterations in the endometrial expression of 11β-HSD enzymes in response to GCs during decidual transformation may be involved in the regulation of several cellular events in addition to modulating GC availability. These include proliferation, apoptosis and the bio-synthesis of hormones, growth factors and enzymes such as matrix metalloproteinases

(MMPs) (McDonald et al, 2006). However, it remains to be determined whether elevated or suppressed levels of either 11β-HSD isoform are associated with fertility or endometrial pathology.

To gain further understanding of the effect of GC exposure and the GR signalling pathway during decidual transformation it was decided to treat ESCs with RU486 (also known as mifepristone), which is known to be a potent non-selective antagonist of GCs (Narvekar et al, 2006). Reviews of the literature revealed that low-dose treatment with RU486 resulted in some alterations in the endometrial expression of the sex steroid hormone receptors, namely PR and AR (Narvekar, 2004). Therefore it was crucial to identify whether treatment of ESCs during decidual transformation had any significant effect on the expression levels of GR, ER, PR or AR (figure 4.18). At the 0.5h time point, it was found that there was a significant reduction in endometrial PR expression within the stromal cells treated with RU486 compared to the control cells (untreated), which is in accordance with previous findings (Narvekar et al, 2006). However, previous literature reports seeing a significant increase in the endometrial expression of AR due to RU486 treatment, which was not observed in this study (Narvekar et al, 2004). This may be attributable to the fact that Narvekar and colleagues carried out much longer treatment durations compared to the longest time point in the experiments presented in this chapter being 2d. It was of great interest to this study, that treatment with RU486 for 48h resulted in a significant decrease in the endometrial stromal cell expression of GR, which was not anticipated. As previous results have suggested that treatment with RU486 either results in GR expression remaining unchanged or an induction of GR expression in the endometrial glandular compartment (which was not under investigation in this chapter) (Narvekar et al, 2006). It was decided to investigate the effect of RU486 at a 48h time point due to the apparent inhibition of GR and the un-altered levels of the sex steroid hormones observed (figure 4.18).

Using RU486 as a GC antagonist, it was found that antagonising the GC effect on the GR signalling pathway results in a reversal of the detrimental effects of cortisol during decidual transformation, in terms of ESC morphology and the expression of the key decidual marker; PRL (figure 4.19 and 4.20). This provides encouraging evidence that the GR signalling pathway is crucial within decidual transformation and the presence of the stress hormone; cortisol is able to activate the GR signalling pathway resulting in alterations in the ability of 'normal' fertile endometrium to undergo successful decidual transformation. This effect was further observed in ESCs undergoing decidual transformation in the presence of cortisone, where the addition of the GR antagonist resulted in the successful morphological transition of the ESCs to the more rounded epithelial-like phenotype (figure 4.21) and the induction of the key decidual marker; dPRL (figure 4.22). Surprisingly, the addition of RU486 and

cortisol during decidual transformation of the ESCs did not seem to have any significant effect on the expression levels of IGFBP-1 at the secreted protein level. Future work to identify the endometrial expression levels of IGFBP-1 at the mRNA level will allow us to further understand the differences in the regulation of dPRL and IGFBP-1 by the GCs. Even though the studies using RU486 provided us with useful information regarding this study, it is important to note that RU486 is also a potent antagonist of progesterone, and is also known to alter endometrial function (Narvekar et al, 2006). This highlights the need of either finding a selective antagonist of GR or by using GR knock-out models which will allow us to fully confirm whether it is the GR signalling pathway which is having a direct effect on the endometrial decidual response.

In addition to investigating the effect of antagonising the GC activation of the GR signalling pathway on key decidual markers, it was also decided to investigate if this antagonistic effect had any significant effect on the expression levels of key proteins involved in the GR signalling pathway, namely GRα itself and the 11β-HSD isoforms. GRβ was excluded from this study, due to its reduced ability to bind GCs. Similar results were found for both the HESC cell line and ESCs isolated from fertile patients, further indicating that the HESC cell line can be used as a suitable model in this study and also in future studies. As previous studies have suggested, antagonising the GCs does not seem to have any significant effect on endometrial stromal GR expression at the protein level, which is fully supported by the results presented in figure 4.23. Interestingly, novel results indicate that antagonising the action of the two GCs under investigation; cortisol and cortisone results in a significant reduction of 11β-HSD1, suggesting that antagonising GC action in ESCs also results in the reduction of the enzyme responsible in converting the inactive cortisone to the active cortisol ligand. This is supported by experiments carried out in mice, which also demonstrated that treatment of hepatocytes with RU486 resulted in decreases in 11β-HSD1 expression (Liu et al, 2005). No effect was observed on the endometrial stromal expression of 11β-HSD2 in response to RU486 treatment, either in the presence or absence of GCs during decidual transformation. The experiments conducted with the GC antagonist; RU486 demonstrates a specific effect of GCs, particularly cortisol, on the ESCs during decidual transformation.

The work presented in chapter 3 and chapter 4 brings to light a possible involvement of the GR signalling pathway as a determining factor of fertility status in females. However, to this point, we still were uncertain whether GR was having a direct effect on crucial genes and proteins within the human endometrium.

GR binds as homodimers specific sequences within the promoter regions of the target genes known as GREs (Vandevyver et al, 2013). Recent global ChIP-seq analysis has shown that GR binds to DNA primarily via the GRE consensus motifs or GR can also co-operate with

other TFs by indirect tethering (Karmakar et al, 2013). In addition to GREs, it is also known that GR is also able to bind negative GREs (nGREs) (Vandevyver et al, 2013).

Initial investigations were conducted to identify potential TF binding sites on the promoter of the NR3C1 gene which encodes GR. Due to the vast amount of data created by the TRANSFAC database, it was necessary to undergo data mining to identify TF binding sites of particular interest. TFs which play a significant role within the endometrium were focused on. TRANSFAC analysis of the identified GR promoter region revealed that the promoter region contained binding sites for numerous TFs which included cAMP-responsive element binding protein (CREB), the Wilms Tumour suppressor (WT1), GR, PR and also AR (table 4.1). The identification of a CREB and PR binding site within the NR3C1 promoter was of particular interest in this study, due to the exposure effect of cAMP on GR mRNA expression levels during in vitro decidualization (figure 4.7).

The identification of the sex steroid hormone receptor binding sites within the GR promoter is in accordance with previous literature. Studies have been conducted which have identified the regulatory effects of GR and the potential crosstalk between GR and the sex steroid hormone receptors. It is known that the amino acid sequence of GR and AR for example, is highly homologous within their DNA binding domains and over 50% of their targeted genes can be commonly regulated by both receptors. Previous work has identified using ChIP and DNA sequencing that the GR promoter contains a negative androgen responsive element (nARE), which AR is able to interact directly with and could mediate transcription repression by ligand activated GR in prostate cancer cells (Xie et al, 2015). This therefore, suggests that GR expression can be negatively regulated by the AR signalling pathway. Additionally, ChIP experiments have shown that GR is recruited to the ERα binding regions in the presence of dexamethasone and estradiol in the MCF-7 cell line. This then leads to the destabilisation of the ER transcriptional complex and protein-protein interaction studies have shown that GR directly interacts with ERα through its DNA-binding domain (Karmakar et al, 2013). Furthermore, crosstalk between GR and PR has also been suggested within breast cancer cells, therefore supporting the identification of PR binding sites within the GR promoter (Leo et al, 2004). Furthermore, negative GREs and potential CREB binding motifs have also been identified within the GR promoter (Govindan, 2010).

The promoter of potential GR-regulated target genes which may a significant role in the GR signalling pathway or within the decidual transformation process in ESCs was also analysed. In-depth promoter analysis of these candidate target genes provided us with encouraging evidence that GR may be able to transcriptionally regulate NR3C1, AR, PGR, PRL, FKBP4, FKBP5, WT1, HSD11B1 and HSD11B2 due to the presence of potential GR binding sites within their promoter regions (refer to table 4.4 for in-depth promoter analysis).

Given the results obtained from the TRANSFAC analysis and previous molecular work in this study, the direct regulation of GR on these target genes was investigated using ChIP analysis. ChIP was used to determine the site specific GR occupancy of the promoters of NR3C1, PRL, WT1, FKBP5, PGR, AR, HSD11B1 and HSD11B2 following 48h treatment of the HESC cell line with either cAMP, cAMP + F, cAMP + E, E, F or RU486 and compared to untreated cells. It must be noted that only one potential GR binding site was chosen for investigation in each target promoter due to time and experimental constraints.

Novel and interesting results were obtained with ChIP analysis. It was elucidated that there were significant increases in the enrichment of GR binding on specific GR binding sites in the promoter regions of PRL (figure 4.26), WT1 (figure 4.27) and FKBP5 (figure 4.28) particularly during in vitro decidualization in response to cAMP treatment either in the presence of absence of GCs (either F or E). This indicates that the GR signalling pathway may be having a direct transcriptional effect on these master regulators of decidualization, providing further supporting evidence of a role of the stress, GR and decidualization in stromal compartment of the endometrium.

In addition to identifying whether GR directly modulates the expression of key genes involved in the decidual response, it was also decided to investigate whether GR was enriched on potential GRE sites on the promoter regions of the two enzymes responsible for controlling the bio-availability of cortisol. It was found that GR enrichment was increased during treatment of the HESC cells with cAMP + E, whereas all other treatments resulted in an overall decrease in GR enrichment compared to control cells (figure 4.31). GR also showed enrichment on the GRE sites on the 11BHSD2 promoter during cAMP stimulation in the presence of absence of GCs (figure 4.32).

The direct GR regulation of AR expression was also assessed, primarily due to the role of WT1 in regulating AR (Gonzalez et al, 2012). Interestingly, this study identified active GRE sites within the AR promoter (figure 4.30), where treatment with cAMP, cAMP + F and cAMP + E resulted in an increase in GR enrichment at the GRE binding site.

Finally, it was also assessed whether PGR was also directly regulated by GR, given that progesterone and progesterone synthetic derivatives are known to act via GR to alter the transcription of target genes and therefore may play role in local GC bio-availability (Lei et al, 2012). It was confirmed that GR was recruited to the GRE elements of PGR (figure 4.29), suggesting an intrinsic regulatory mechanism within the human endometrium that needs further investigation.

The ChIP data presented in this chapter allows us to form the hypothesis that GR plays a significant role in the modulation of genes involved in the decidual transformation of ESCs,

particularly in response to cAMP in the presence of absence of GCs. Due to the apparent effect of cortisol and the GR signalling pathway on the regulation of decidualization, then it is of further interest to elucidate GR expression and its response to ligands in ESCs isolated from infertile patients. Additionally, further work is needed to identify potential GR targets in infertile endometrium.

CHAPTER 5

The effect of sex steroid hormones and the GR signalling pathway on decidual transformation of the human fertile and infertile endometrium

5.1 Introduction

It is estimated that infertility affects one in every six heterosexual couples in the UK, where 30% of infertility cases are attributed to male factors, and the remaining 70% are recognised as female factors. These female factors include ovulatory disorders, uterine disorders or can merely be 'unexplained' (NICE, 2012). The majority of infertility cases are a result of well-characterised pathologies which include PCOS and endometriosis.

PCOS is a common endocrine disorder which affects 6-10% of women of reproductive age. PCOS is characterised by a broad spectrum of clinical manifestations which vary in severity. These include oligo-menorrhea, infertility and hyperandrogenism (Lopes et al, 2011). PCOS-related infertility has been associated with elevated androgen levels present locally within the endometrium and circulation, resulting in high endometrial expression of AR, which may have a significant impact on endometrial decidual transformation (Gonzalez et al, 2012). The clinical manifestations of PCOS can implicate a significant psychological burden on the patients suffering from this syndrome (Stefanaki et al, 2015). On the other hand, endometriosis is a debilitating chronic inflammatory disorder which also affects 6-10% of women of reproductive age and 25-50% of infertile women (Bulletti et al, 2010; Hickey et al, 2014; Monsivais et al, 2016). Endometriosis is also associated with endometrial progesterone resistance which can lead to a pro-inflammatory environment and impaired endometrial decidual transformation (Patel et al, 2017). UI is diagnosed when all standard diagnostic tests used to identify infertility fail to identify any reproductive pathology which may be causing the infertility (Lindsay et al, 2015).

In addition to the clinical manifestations of these reproductive disorders, it is known that those suffering from either PCOS or endometriosis can suffer from severe psychological symptoms, which include higher levels of perceived stress and anxiety which can be damaging to their health (Cuevas et al, 2012; Hart and Doherty, 2015; Barthelmess and Naz, 2014; Hickey and Farquhar, 2014).

In addition to reproductive pathologies, many infertile females are suffering from unexplained infertility (UI). These patients are not affected by a specific etiology or do not present with any endometrial or ovulatory defect (Jose-Miller, Boyden and Frey, 2007). However, several studies have identified that stress and anxiety may be contributing to their infertility and may also be contributing to whether ARTs are successful in these patients (Donarelli et al, 2016).

It has been reported before that GR can interact with the ER nuclear receptors in breast cancer cells (Karmakar et al, 2013) and more recently endometrial cancer cells (Vahrenkamp

et al, 2018). In addition, GR is also able to crosstalk with PR in breast cancer cells (Leo et al, 2004). Recent studies have also elucidated that GR expression is able to be modulated by active AR signalling in prostate cancer and moreover, AR and GR can commonly regulate the majority of their target genes (Xie et al, 2015). Additionally, it has been proven that GR up-regulation in prostate cancer can confer resistance to androgen hormone therapy by allowing androgens to bypass AR and instead have an effect through the GR signalling pathway (Arora et al, 2013). Thus, it would be important to elucidate if GR modulates the effects of PR and AR in the human endometrium during decidual transformation.

The relationship between the hormonal and metabolic profiles and the psychological symptoms observed in infertile patients remains debatable. However, due to the high prevalence of psychological disorders exhibited in these patients, it is important to conduct more research to reveal the role of the GR signalling pathway and potential crosstalk with other sex steroid hormone receptor signalling pathways in the development of these disorders.

Previous work in this study has identified a differential expression of endometrial GR and the key enzymes involved in controlling cortisol bio-availability in biopsies isolated from both fertile and infertile patients at specific stages of the menstrual cycle. Furthermore, the active endometrial GR signalling pathway may have a significant role during endometrial decidual transformation in the fertile patients by directly modulating the expression levels of key genes via binding to GREs present on the promoter sites of these target genes.

Therefore, this chapter focuses on expanding the work conducted in chapter 3 and 4 by aiming to determine the endometrial stromal expression of GR and the effect of the GR signalling pathway in the presence and absence of the stress hormones during decidual transformation in the infertile endometrium. It is known that defects in decidual transformation can exist in patients suffering from PCOS and endometriosis; therefore it is of great interest to see if there are unique differences in GR signalling in these patients. This will be conducted using ESCs isolated from infertile patients, instead of using whole tissue biopsies to elucidate effects on the stromal compartment. Furthermore, this chapter will also hope to shed light on whether altered sex hormone receptor signalling pathways present in these infertile pathologies, namely AR and PR and their corresponding ligands, DHT and P4, have any significant effect on the GR signalling pathway during decidual transformation in the presence and absence of the stress hormone.

This chapter will present preliminary work carried out using ESCs isolated from patients suffering from UI. This work aimed to identify basal GR expression in the stromal

compartment and identify the effect of stress hormone exposure on the GR signalling pathway during in vitro decidual transformation.

Work presented in this chapter aims to shed light on whether stress exposure and the activation of the GR signalling pathway has a significant effect on common infertile pathologies.

5.2 Results

5.2.1 Basal expression of the glucocorticoid receptor (GR) and 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes in endometrial stromal cells isolated from infertile patients

As previously mentioned, the expression of GR and 11β-HSD1 has been previously described within the human endometrium in this study and by previous work conducted by Bamberger and colleagues (GR only) (Bamberger et al, 2001). However, little research has been conducted to elucidate the expression of GR and the 11β-HSD enzyme during the menstrual cycle in infertile patients, particularly those suffering from PCOS, endometriosis and unexplained infertility (UI).

Therefore, initial experiments were conducted to determine the basal protein and gene expression levels of GR and the 11β-HSD enzymes in endometrial stromal cells (ESCs) isolated from infertile patients. Infertile patients included those suffering from ovulatory PCOS (ovPCOS), anovulatory PCOS (anPCOS), endometriosis and UI.

Initial results showed that ESCs isolated from both anPCOS and ovPCOS have a significantly higher basal expression of GRα compared to ESCs isolated from fertile patients ($p \leq 0.05$) (figure 5.1A and B). Whereas ESCs isolated from endometriosis and UI patients showed no significant difference in the basal protein expression levels of GRα compared to fertile patients. Interestingly, patients suffering from anPCOS and ovPCOS showed a significant reduction of GRβ expression levels compared to fertile patients ($p \leq 0.05$) (figure 5.1A and B). Whereas similar to results obtained for GRα, endometriosis and UI patients showed no difference in the expression levels of GRβ compared to fertile patients. Immunoblot analysis was also carried out for the 11β-HSD enzymes, which demonstrated that ESCs isolated from both anPCOS and endometriosis patients have higher basal expression levels of the type 1 isoform; 11β-HSD1 compared to fertile patients ($p \leq 0.05$) (figure 5.1A and C).

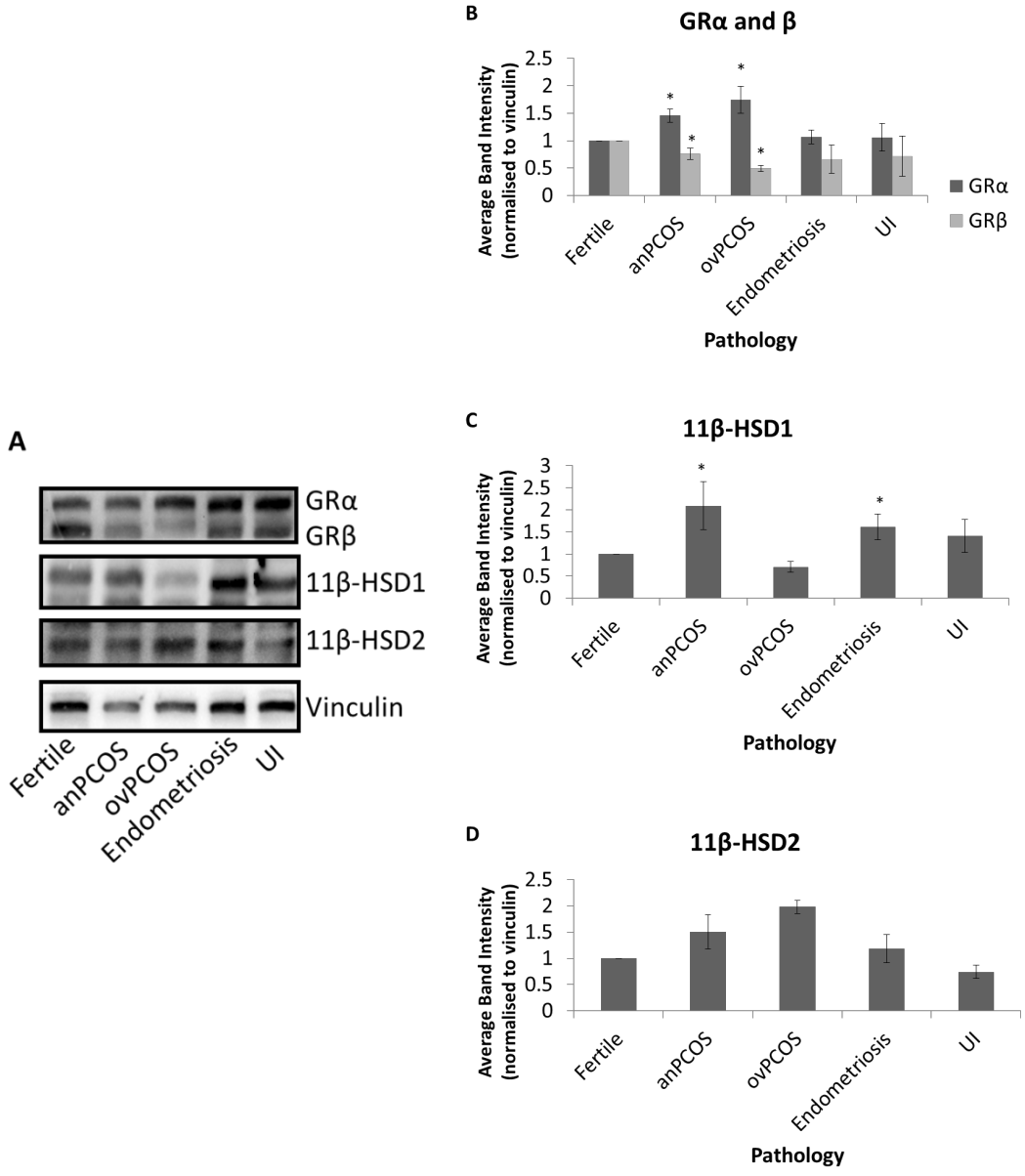


Figure 5.1A-D. Basal protein expression levels of GR and 11βHSD in ESCs isolated from infertile patients suffering from either anovulatory PCOS (anPCOS), ovulatory PCOS (ovPCOS) endometriosis or unexplained infertility and compared to fertile patients. A) Image shows the representative images of western blot analysis of GRα (95kDa), GRβ (90kDa), 11βHSD1 (32kDa) and 11βHSD2 (40 kDa) protein levels in ESCs isolated from fertile patients (n=5), anPCOS patients (n=5), ovPCOS patients (n=3) and endometriosis patients (n=6). B-D) The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the house keeper protein; vinculin (124 kDa). Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

Conversely, ovPCOS and UI patients express similar levels of 11β-HSD1 compared to fertile patients. Furthermore, immunoblot analysis showed that ESCs isolated from anPCOS and ovPCOS patients have an on average higher expression of 11β-HSD2 protein compared to fertile patients (not significant). Endometriosis exhibit similar expression levels of 11β-

HSD2 when compared to the fertile patients and ESCs isolated from UI patients express lower levels of the type 2 isoform compared to fertile patients (not statistically significant).

qPCR analysis was also carried out using gene specific primers to determine whether basal mRNA expression levels of GR, 11β-HSD1 and 11β-HSD2 reciprocated the basal levels of protein seen within the ESCs. It was found that the basal mRNA levels of GR did indeed replicate the basal protein levels seen in figure 5.1. It was found that ESCs isolated from anPCOS and ovPCOS patients had significantly higher levels of basal GR mRNA compared to fertile patients ($p \leq 0.05$) (figure 5.2A), whereas no differences in basal mRNA expression levels were found for GR mRNA levels between either endometriosis patients or UI patients when compared to fertile patients. It is important to note that the gene specific primers designed to recognise GR, recognise both α and β isoforms. Similar to that seen at the protein level, qPCR analysis revealed that anPCOS and endometriosis patients had a significantly higher basal expression of 11β-HSD1 at the mRNA level compared to fertile patients ($p \leq 0.05$), whereas no differences in the expression levels were seen between either ovPCOS or UI patients compared to fertile patients (figure 5.2B). The basal gene expression levels of 11β-HSD2 also followed a similar pattern to that seen at the protein level. It was found that ESCs isolated from patients suffering from anPCOS and ovPCOS had a significantly higher basal expression of 11β-HSD2 at the mRNA level compared to fertile patients ($p \leq 0.01$ and $p \leq 0.05$ respectively) (figure 5.2C). Additionally, ESCs isolated from endometriosis and UI patients displayed no significant alterations in the expression of the type 2 isoform at the mRNA level (figure 5.2C).

This data therefore suggests that ESCs isolated from infertile patients (anPCOS, ovPCOS and endometriosis) express both isoforms of GR. Endometrial expression of GRα in the stromal compartment of anPCOS and ovPCOS patients is significantly higher than that observed within fertile patients. Whereas similar levels of endometrial GRα expression are seen in both endometriosis and UI patients compared to fertile patients. However, immunoblot analysis reveals that there seems to be alterations in the basal expression levels of GRβ in ESCs isolated from infertile patients compared to fertile patients.

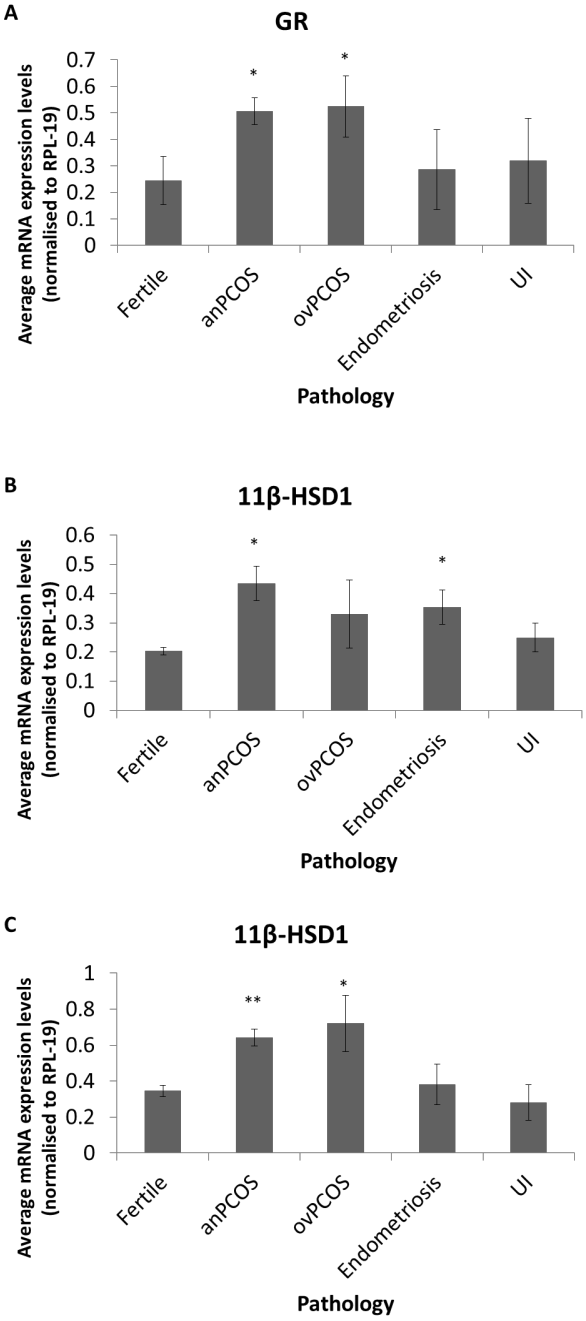


Figure 5.2. Basal mRNA expression levels of GR and 11βHSD in ESCs isolated from infertile patients suffering from anPCOS, ovPCOS, endometriosis or UI and compared to fertile patients. A-C) Graph shows the basal GR (A), 11β-HSD1 (B) and 11β-HSD2 (C) mRNA levels in ESCs isolated from fertile patients (n=5), anPCOS patients (n=5), ovPCOS patients (n=3), endometriosis patients (n=6) and UI patients (n=3) by qPCR analysis. Values shown are the mean starting quantity from triplicate values per sample, normalised to the housekeeper gene; RPL-19. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

Furthermore, ESCs isolated from infertile patients also express both 11β-HSD enzymes within the endometrial stroma, which allow the ESCs to participate in the previously mentioned cortisol-cortisone shuttle. However, even though ESCs isolated from infertile patients seem to express both 11β-HSD1 and 11β-HSD2, there are significant differences in basal protein and mRNA expression compared to fertile patients in some patient cohorts (figure 5.1 and 5.2). This preliminary data suggests that differences in the expression of key genes and proteins involved in the GR signalling pathway may have a significant effect on the development of these infertile pathologies, which warrants further investigation.
Therefore, it was decided the biologically active ligand cortisol and its inactive counterpart, cortisone will be used to determine the effect of stress exposure and the activation of the GR signalling pathway in ESCs isolated from infertile patients (either ovPCOS, anPCOS or endometriosis). UI patients were excluded from these studies initially.

5.2.2 The effect of cortisol during decidual transformation of endometrial stromal cells isolated from infertile patients

As seen in the fertile patients described in chapter 4, the ESCs isolated from the infertile patients expressed GR and the 11β-HSD enzymes, which are crucial in controlling the local bioavailability of the stress hormone cortisol. It was therefore decided to investigate the role of cortisol and the GR signalling pathway in these patients during the crucial decidualization process.

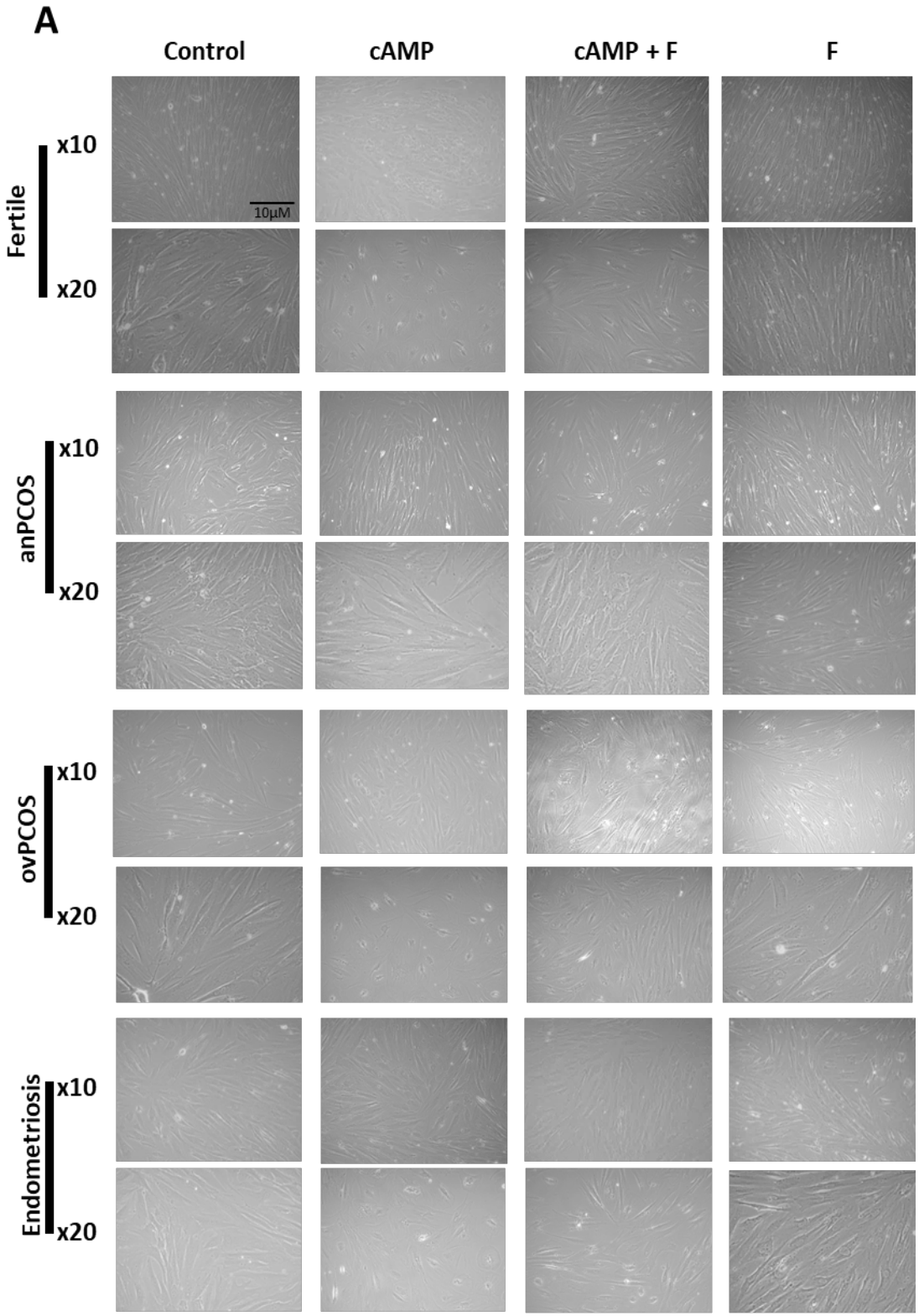
In order to investigate the role of cortisol and the GR signalling pathway on *in vitro* decidual transformation of ESCs isolated from primary tissue, the isolated cells were cultured and treated with the *in vitro* decidualization stimulus; cAMP, in combination with the stress hormone cortisol for 48h.

As described in section 4.2.2, decidualization was determined based on the morphological appearance and changes in the ESCs and also by the expression of key decidual markers. This therefore comprised of monitoring a change in shape from a fibroblast shaped cell to a more rounded epithelial like cell and additionally the induction of the expression of dPRL and IGFBP-1.

5.2.2.1 The effect of cortisol on cell morphology and the expression of key decidual markers during decidual transformation in infertile patients

Cell monolayers were treated for 48h with cAMP, cAMP + F or F alone and compared to untreated control cells. It was found that treatment with the *in vitro* decidualization stimulus; cAMP, resulted in a significant change in cell morphology, where ESCs isolated from all patient groups adopted a more rounded epithelial like phenotype to varying extents, compared to the normal fibroblast phenotype that stromal cells exhibit (figure 5.3 A and B). This change in morphology is demonstrated visually in figure 5.3A, where it can be seen that after treatment with cAMP for 48h, the ESCs isolated from fertile patients have undergone successful decidual transformation *in vitro*. Exposure to the decidualization stimulus also resulted in a change in morphology of the ESCs isolated from the infertile patients, but this was to a lesser extent. These visual changes were further confirmed quantitatively using Image J analysis, where it was found that ESCs isolated from fertile patients treated with cAMP had a significant increase in the their circularity values compared to the control cells, where this value increased on average from approximately 0.25 to 0.70 ($p \leq 0.001$) (figure 5.3B). As previously mentioned, the ESCs isolated from infertile patients also had a change in morphology, which was also confirmed with Image J analysis. Treatment with cAMP resulted in an increase in the circularity values from approximately 0.2 to 0.38, 0.2 to 0.50 and 0.24 to 0.55 for the ESCs isolated from anPCOS, ovPCOS and endometriosis patients respectively ($p \leq 0.05$, $p \leq 0.01$, $p \leq 0.01$) (figure 5.3B). However, even though a successful change in cell morphology has taken place in these infertile patients, this process seems to be impaired, as all three patient groups have a lower cell circularity value compared to ESCs isolated from fertile patients.

As mentioned in chapter 4, an untreated ESC isolated from primary tissue has an average circularity value of approximately 0.2, which remains consistent whether the patient is fertile or is suffering from PCOS or endometriosis and is therefore infertile. ESCs isolated from fertile patients that have undergone successful decidualization have an increased circularity value ranging from 0.5-1.0, where a value of 1.0 indicates a perfect circle. The results presented in figure 5.3 suggest that *in vitro* decidualization success rates are not comparable between ESCs isolated from fertile patients and those isolated from infertile patients.


A
Control
cAMP
cAMP + F
F
Fertile
x10
x20
10µM
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ovPCOS
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Endometriosis
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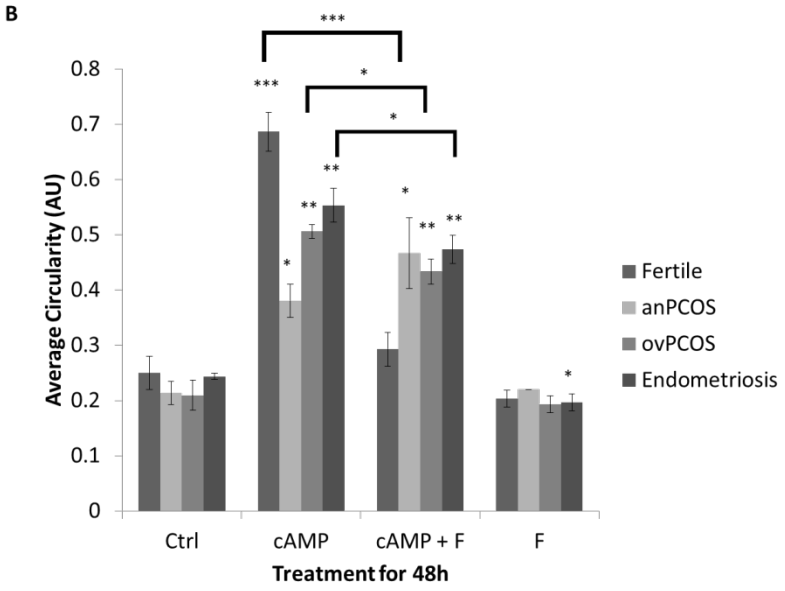


Figure 5.3A and 5.3B. The effect of cortisol (F) on the morphology and circularity values of ESCs isolated from infertile patients suffering from anPCOS, ovPCOS or endometriosis and compared to fertile patients during decidual transformation. A) Representative images taken of the cell monolayers after 48 h of treatments using inverted light microscopy (Zeiss) at an x10 objective and an x20 objective. These images were then used for Image J analysis to gain a quantitative measure of cell morphology. B) Thirty cells were chosen at random from each image taken of ESCs isolated from fertile patients (n=5), anPCOS patients (n=5), ovPCOS patients (n=3) and endometriosis patients (n=6). Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

As discussed in chapter 4 – the presence of the stress hormone cortisol appears to impair successful decidualization taking place in the fertile patients. Interestingly, it was demonstrated that co-treatment with cAMP and F caused an overall increase in the average cell circularity values in ESCs isolated from anPCOS patients compared to those treated with cAMP alone (not statistically significant). Whereas co-treatment with cAMP and F resulted in a decrease in the cell circularity values obtained for ESCs isolated from ovPCOS and endometriosis patients (p≤0.05). Due to the small alterations of cell morphology, these changes are not clearly visible in the images displayed in figure 5.3A. As seen with the fertile patients, treatment with F alone resulted in no significant difference in the average circularity values obtained compared to untreated control cells in ESCs isolated from PCOS patients, however a significant reduction in circularity values was seen in ESCs isolated from endometriosis patients (p≤0.05) (figure 5.3B).

To further support the morphological changes seen in these cells – the concentration of key secreted decidual markers was measured using commercially available ELISA kits.

It was found that treatment of the ESCs isolated from all infertile patients (PCOS and endometriosis) with cAMP alone and co-treatment with cAMP and F caused a significant increase in the concentration of secreted prolactin compared to the control untreated cells ($p \leq 0.05$) (figure 5.4A). Even though, treatment of the ESCs isolated from infertile patients with cAMP caused an increase in the secretion of prolactin, there was a notable reduction in these levels compared to the fertile patients which undergo successful decidual transformation. For example, ESCs isolated from fertile patients secrete on average approximately 800 pg/mL compared to only 230 pg/mL which is secreted by ESCs isolated from infertile anPCOS patients. As discussed in chapter 4, co-treatment with cAMP and F results in a significant reduction of the expression and secretion of prolactin compared to cells treated with cAMP only, however these results were not reciprocated in the infertile patients. Alternatively, ELISA analysis suggests that co-treating the ESCs isolated from infertile patients with cAMP and F appears to have no significant effect on the expression and secretion of prolactin compared to cells treated with the *in vitro* decidualization stimulus only (figure 5.4A).

Comparable results were obtained for secreted levels of IGFBP-1 (figure 5.4B). It was found that treatment of ESCs isolated from infertile patients with cAMP alone resulted in a significant increase in the concentration of secreted IGFBP-1 levels ($p \leq 0.05$); however the concentrations were significantly lower than those seen with ESCs isolated from fertile patients. As seen with prolactin, treatment of ESCs isolated from infertile patients with cAMP and F also resulted in a significant increase in the secreted levels of IGFBP-1 compared to untreated cells.

Moreover, ESCs isolated from ovPCOS and endometriosis patients resulted in slight reductions of the secreted concentration of IGFBP-1, but these were not statistically significant. Interestingly, co-treatment with cAMP and F resulted in a significant increase in IGFBP-1 concentration in ESCs isolated from infertile anPCOS patients compared to those treated with cAMP only ($p \leq 0.05$) (figure 5.4B). It is important to note that even though there is a statistically significant induction of the expression and secretion of IGFBP-1 in the infertile patients, the concentrations obtained are significantly lower than those seen in ESCs isolated from fertile patients. This further indicates that decidualization is impaired to varying extents in these patient cohorts.

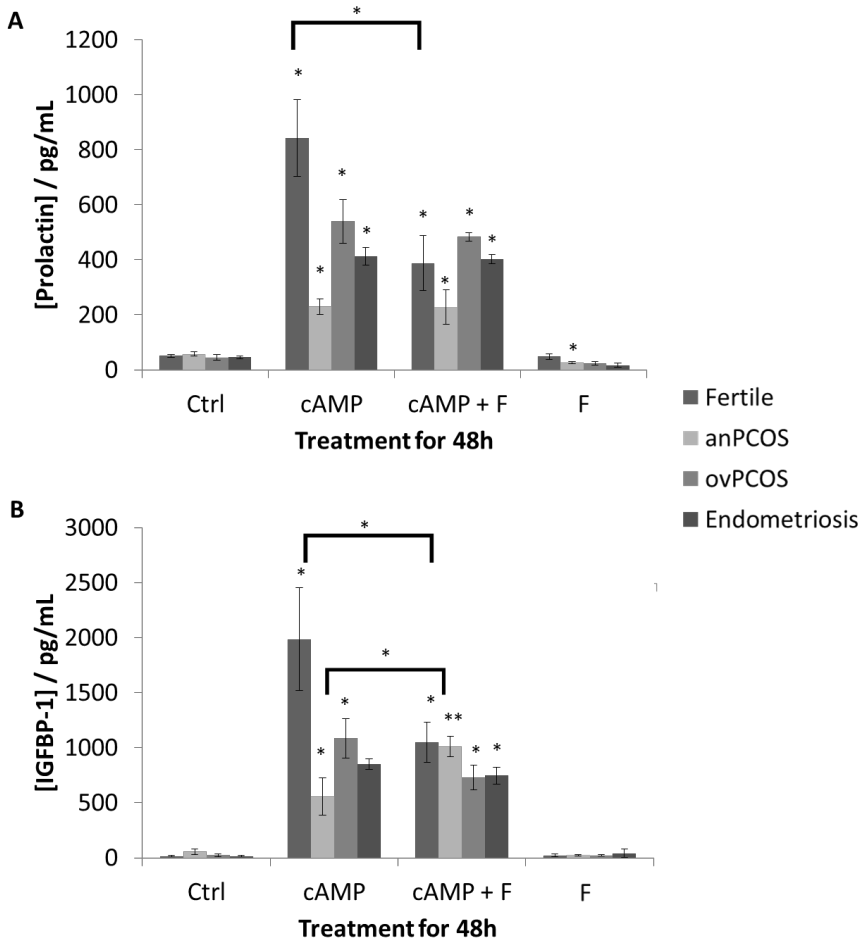


Figure 5.4. The effect of cortisol (F) on the levels of secreted decidual markers – prolactin (A) and IGFBP-1 (B) from ESCs isolated from infertile patients either suffering from anPCOS, ovPCOS or endometriosis and compared to fertile patients during decidual transformation. Media was extracted after ESCs isolated from fertile patients (n=5), anPCOS patients (n=5), ovPCOS patients (n=3) and endometriosis patients (m=6) underwent 48 h of treatment with cAMP, cAMP and F or F alone and compared to the control. ELISA experiments were carried out on the conditioned media to establish the concentration of key proteins at the secreted level. Treatment with cAMP caused a significant increase in the secreted levels of both decidual markers compared to the control in all pathology groups. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

These ELISA results were further confirmed using qPCR analysis to determine the expression levels of dPRL and IGFBP-1 at the mRNA level using gene specific primers.

Analysis of the results indicated that both dPRL and IGFBP-1 expression was induced significantly in all patient groups when treated with cAMP alone (p≤0.05) (figure 5.5A and B), therefore supporting the results obtained using the ELISAs. Additionally, co-treatment with cAMP and F also resulted in an increase in the expression of dPRL and IGFBP-1 at the mRNA level compared to untreated cells, in all patient groups, except for ESCs isolated from anPCOS patients where co-treatment did not result in any significant alteration of the gene expression levels of dPRL compared to untreated cells (figure 5.5A). Interestingly,

unlike the ELISA results, there were no significant differences in the expression of the two key decidual markers in all infertile groups studied which were treated with cAMP and F and compared to those treated with the decidualization stimulus only (figure 5.5A and B). This is similar to mRNA results obtained with the HESC cell line and ESCs isolated from fertile patients, and therefore suggests that cAMP and F treatment may only be significantly altering the secreted levels of the decidual proteins in the ESCs.

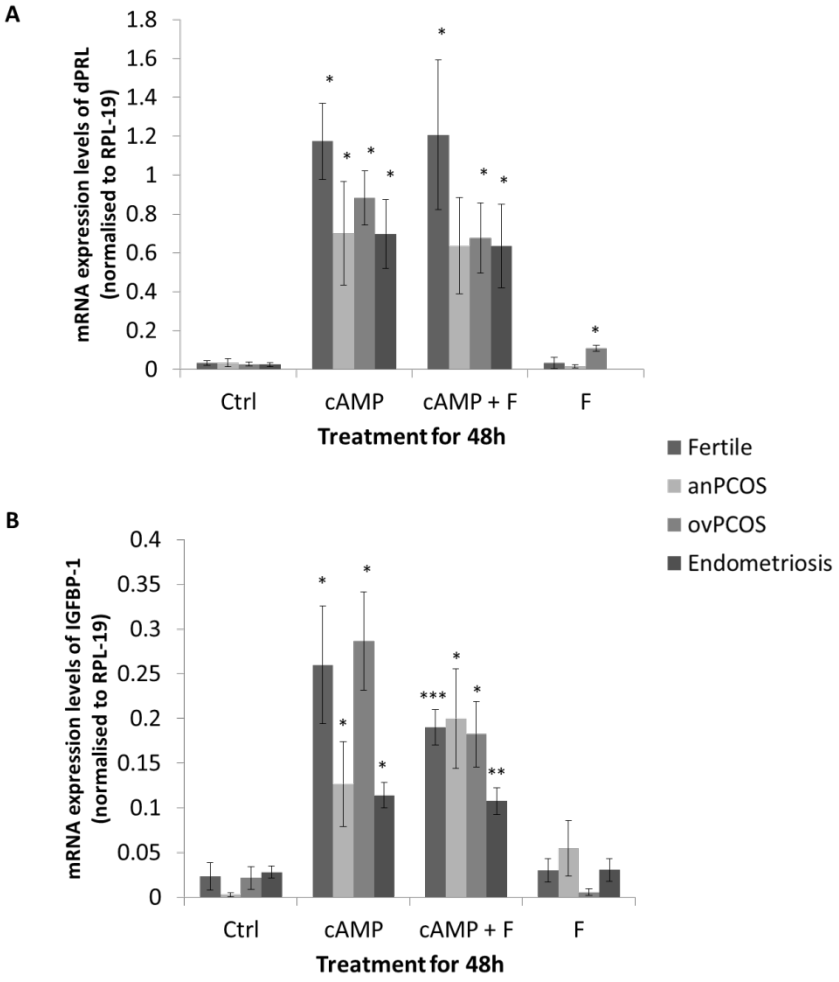


Figure 5.5 The effect of cortisol (F) on the levels of the mRNA expression levels of key decidual markers – decidual prolactin (dPRL) (A) and IGFBP-1 (B) in ESCs isolated from infertile patients suffering from either anPCOS, ovPCOS or endometriosis and compared to fertile patients during decidual transformation. After 48 h of treatment, ESCs isolated from fertile patients (n=5), anPCOS patients (n=5), ovPCOS patients (n=3) and endometriosis patients (n=6) were lysed, RNA extracted and converted to cDNA and used for qPCR analysis. Cells underwent 48 h of treatment with cAMP, cAMP and F or F alone and compared to the control. qPCR analysis was carried out to establish the expression levels at the mRNA level of dPRL (A) and IGFBP-1 (B). Treatment with cAMP alone and cAMP and F, caused an increase in the expression levels of both decidual markers compared to the control in the majority of cases. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

5.2.2.2 The effect of cortisol on the expression of GR and 11β-HSD during decidual transformation in infertile patients

To replicate the experiments carried out on the HESC cell line and ESCs isolated from fertile patients, it was decided that the role of GR and the 11β-HSD enzymes during decidual transformation in the absence and presence of cortisol should also be investigated in the ESCs isolated from infertile patients. As previously described, the ESCs were cultured and treated with cAMP, cAMP + F or F alone for 48h and compared to untreated ESCs isolated from anPCOS patients.

Previous results in this chapter suggest that undifferentiated ESCs isolated from infertile patients express GR to varying extents at the basal level, particularly the less investigated β isoform. Interesting results were observed for the ESCs isolated from infertile patients (PCOS). It was found that *in vitro* decidualization of ESCs isolated from anPCOS patients resulted in no significant difference in the expression of GRα at the protein level compared to untreated cells (figure 5.6A and B), however a significant decrease in the levels of GRβ were observed after decidualization stimulus exposure compared to untreated cells ($p \leq 0.05$) (figure 5.6A and C). Additionally, co-treatment of anPCOS ESCs, with cAMP and F or F alone resulted in a significant decrease in the expression of GRα only and not GRβ compared to untreated cells ($p \leq 0.01$). Experiments were repeated with ESCs isolated from ovPCOS patients and converse results were obtained. In comparison to anPCOS patients, it was found that treatment of ESCs isolated from ovPCOS patients with cAMP resulted in the significant increase of the expression of GRα compared to untreated cells ($p \leq 0.05$) (figure 5.6A and B). Co-treatment with cAMP and F and treatment with F alone, as witnessed in anPCOS patients, seems to on average, result in a decrease in the protein expression levels of GRα, but this was not deemed to be significant.

Further statistical analysis revealed that in both anPCOS and ovPCOS patients, that co-treatment with cAMP and F resulted in a significant decrease in the expression of GRα at the protein level compared to cells treated with the *in vitro* decidualization stimulus only ($p \leq 0.05$ and $p \leq 0.01$ respectively) (figure 4.6A and B). Where similar results were obtained in ESCs isolated from fertile patients (presented in chapter 4). Whereas the opposite effect was displayed for GRβ, where co-treatment resulted in an overall increase in protein expression in anPCOS patients which was not deemed significant and furthermore a significant increase in GRβ expression for ESCs isolated from ovPCOS patients compared to cells treated with cAMP only ($p \leq 0.01$) (figure 5.6A and C).

In addition to GR, previous results in this chapter also suggest that undifferentiated ESCs isolated from infertile patients also express 11β-HSD1 and 11β-HSD2 at the basal level to varying extents. It was found that *in vitro* decidualization of the ESCs isolated from infertile patients (PCOS) resulted in no statistically significant changes in the expression of 11β-HSD1 at the protein level compared to the untreated cells in any treatment group studied (figure 5.6A and D). On average, there seems to be an increase in the expression of 11β-HSD1 expression after cAMP stimulation in anPCOS patients with the reverse effect being seen in ovPCOS patients. ESCs isolated from ovPCOS patients seem to follow a similar trend to that seen with ESCs isolated from fertile patients, which indicate that exposure to cAMP results in a small reduction in the expression of the type 1 enzyme.

On the other hand, western blot analysis showed that 11β-HSD2 protein seems to be aberrantly expressed in ESCs isolated from infertile anPCOS patients, where cAMP appears to induce the expression of this enzyme compared to untreated cells (not statistically significant). This high level expression is maintained when the ESCs are treated with cAMP and F or F alone and compared to untreated cells ($p \leq 0.05$) (figure 5.6A and E). ESCs isolated from ovPCOS patients follow a similar trend to the results obtained from fertile patients, where it is seen that the low expression of 11β-HSD2 protein, which is seen in basal conditions is maintained regardless of treatment, with no alterations in expression taking place.

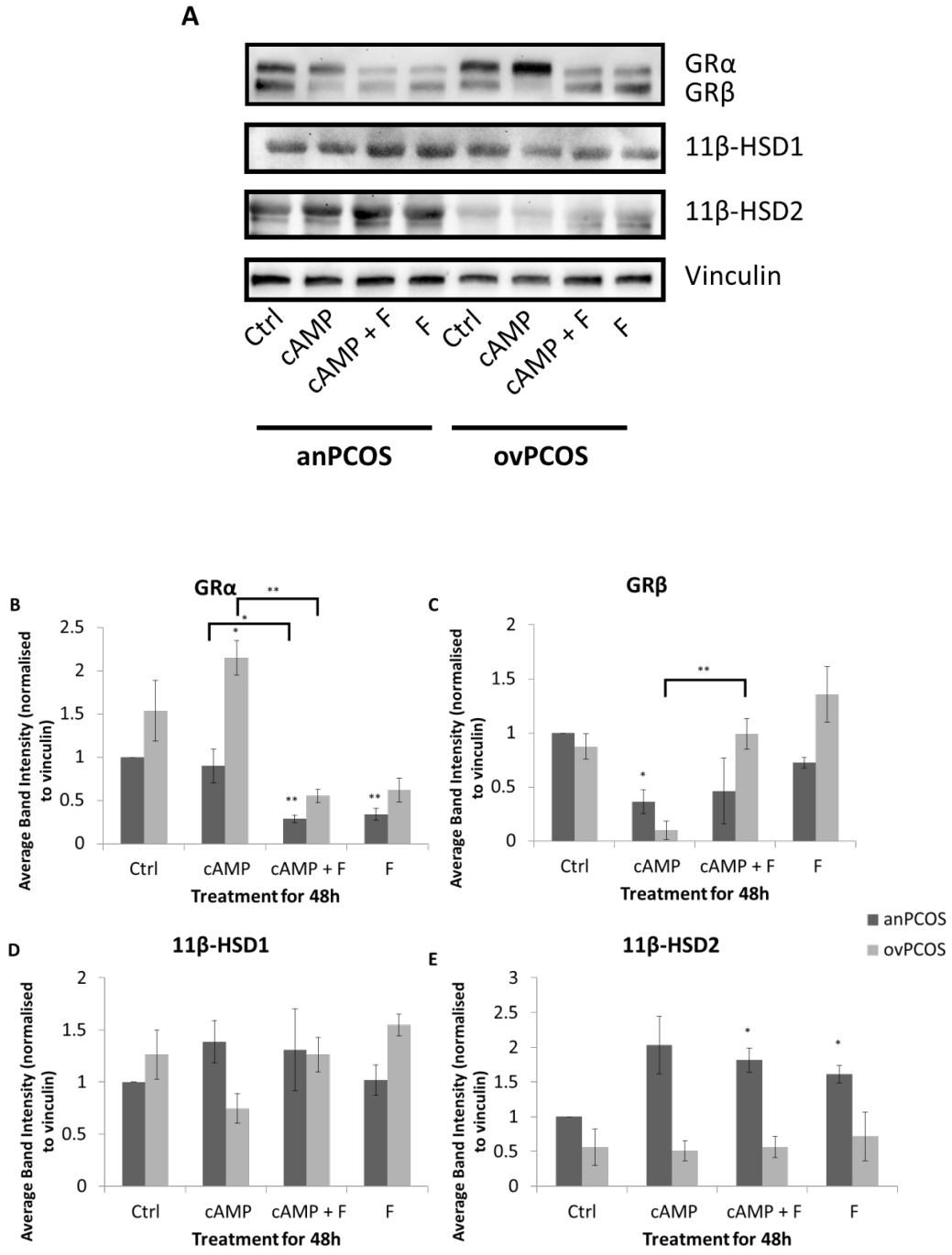


Figure 5.6 The effect of cortisol (F) on key proteins involved in the GR signalling pathway during decidual transformation in ESCs isolated from infertile patients either suffering from anPCOS or ovPCOS. A) Representative images show the western blot analysis of GRα (95kDa), GRβ (90kDa), 11βHSD1 (32 kDa) and 11βHSD2 (40 kDa) protein levels in ESCs isolated from anPCOS patients (n=5) vs ESCs isolated from ovPCOS patients (n=3) after 48h treatment with cAMP, cAMP and F or F alone and compared to the untreated control. B-E) The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the house keeper protein; vinculin (124 kDa). Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

Immunoblot analysis was also carried out on ESCs isolated from endometriosis patients, but analysis had to be carried out separately due to insufficient space on the western blot gels.
It was found that *in vitro* decidualization of ESCs isolated from endometriosis patients resulted in a significant induction of the expression of GRα compared to untreated cells ($p \leq 0.05$) and no overall change in the expression of GRβ (figure 5.7A, B and C), which were similar to results observed with patients suffering from ovPCOS. However, treatment with cAMP and F resulted in ESCs isolated from endometriosis patients following the same trend seen in both PCOS groups studied, where a significant decrease in GRα expression was observed compared to both untreated cells and cells treated with cAMP only ($p \leq 0.05$ and $p \leq 0.001$ respectively) (figure 5.7A and B). Conversely, co-treatment actually resulted in a significant decrease in the expression of GRβ compared to both untreated and cells treated with cAMP only ($p \leq 0.01$) (figure 5.7A and C).

In addition to alterations in GR expression levels, it was found that *in vitro* decidualization of the ESCs isolated from endometriosis patients resulted in no statistically significant changes in the expression of 11β-HSD1 at the protein level compared to the untreated cells in any treatment group studied (figure 5.7A and D), which was also observed in PCOS patients. However, unlike PCOS patients, co-treatment with cAMP and F resulted in a significant decrease in the expression of 11β-HSD1 compared to cells stimulated with cAMP only ($p \leq 0.05$). ESCs isolated from endometriosis patients follow a similar expression pattern to ovPCOS and fertile patients where a low expression of 11β-HSD2 protein, which is seen in basal conditions is maintained regardless of treatment. However, unlike ovPCOS patients, treatment with cAMP results in a statistically significant induction of 11β-HSD2 expression compared to untreated cells ($p \leq .05$) (figure 5.7A and E).

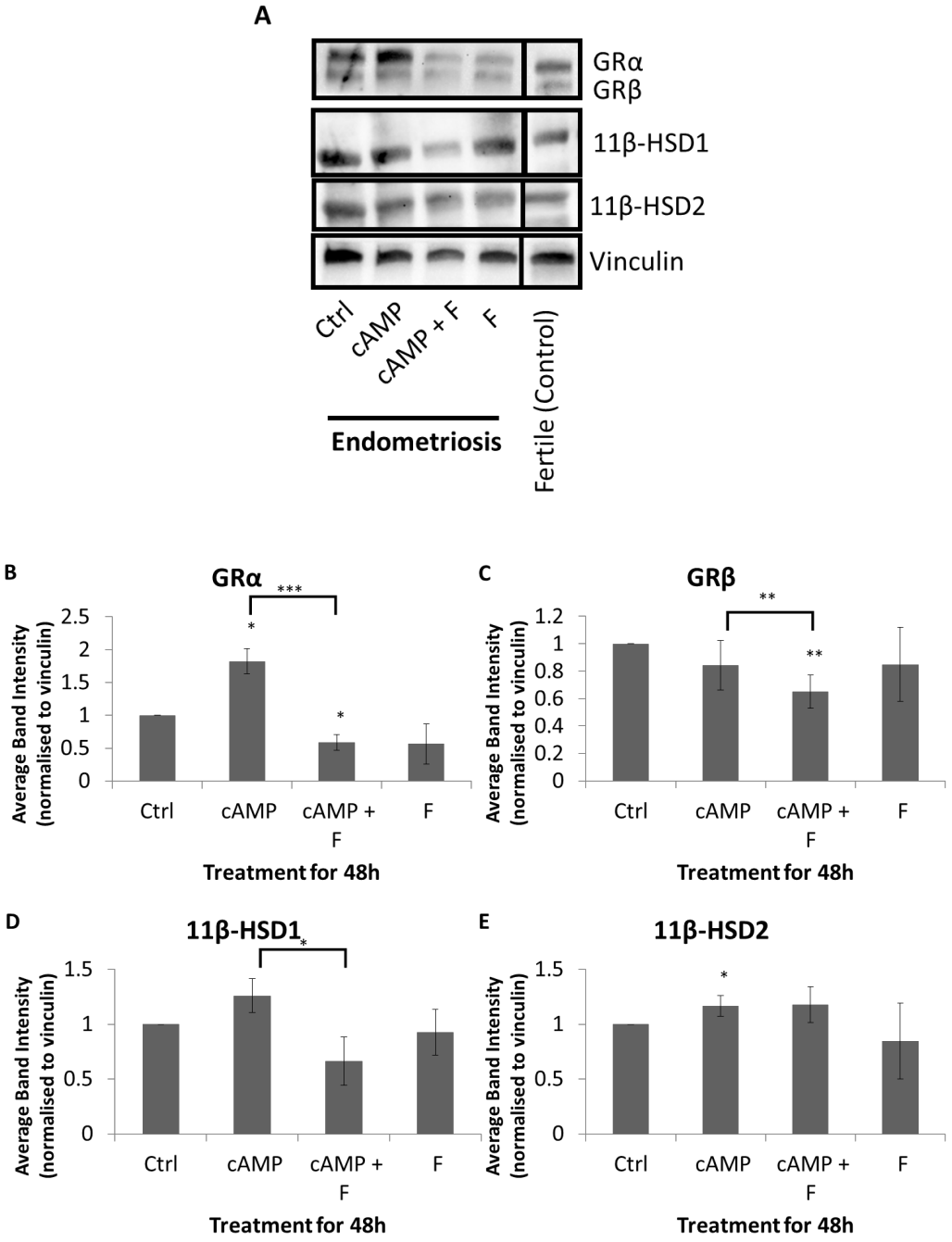


Figure 5.7 The effect of cortisol (F) on key proteins involved in the GR signalling pathway during decidual transformation in ESCs isolated from infertile patients suffering from endometriosis. A) Representative images show the western blot analysis of GRα (95kDa), GRβ (90kDa), 11βHSD1 (32 kDa) and 11βHSD2 (40 kDa) protein levels in ESCs isolated from endometriosis patients (n=6) after 48h treatment with cAMP, cAMP and F or F alone and compared to the untreated control. Untreated ESCs isolated from a fertile patient was also included as a reference for basal levels. B-E) The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the house keeper protein; vinculin (124 kDa). Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

Preliminary results using qPCR investigated whether GR and MR expression was altered during decidual transformation *in vitro* in infertile patients, where it was found that infertile patients seem to express higher levels of GR during decidual transformation compared to fertile patients and lower levels of MR compared to fertile patients (figure 5.8). This suggests that the switch from GR to MR activation in ESCs isolated from fertile patients during decidualization does not take place in infertile patients.

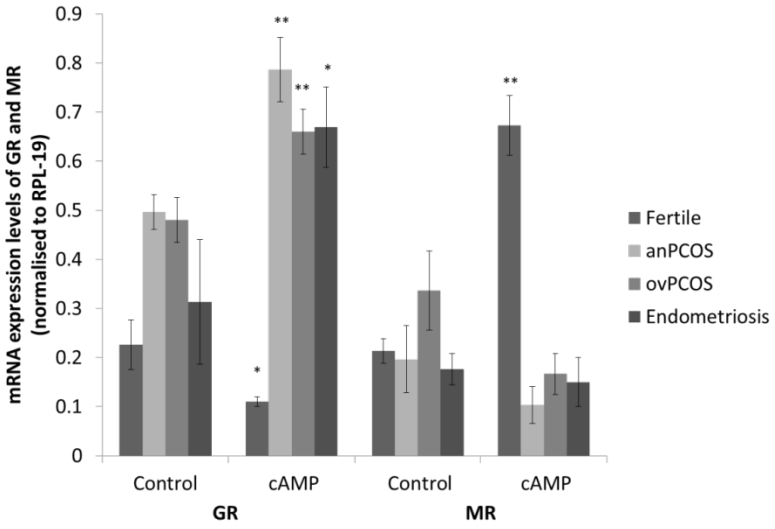


Figure 5.8 Preliminary results showing the effect of in vitro decidualization on the levels of mRNA of GR and MR in ESCs isolated from infertile patients either suffering from anPCOS, ovPCOS or endometriosis and compared to fertile patients. After 48 h of treatment, ESC isolated from fertile patients (n=5), anPCOS patients (n=5), ovPCOS patients (n=3) and endometriosis patients (n=6) were lysed, RNA extracted and converted to cDNA and used for qPCR analysis. Cells underwent 48 h of treatment with either cAMP or were left untreated. qPCR analysis using gene specific primers was carried out to establish the expression levels at the mRNA level of GR and MR. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

5.2.2.3 The effect of cortisol on the expression of potential GR targets during decidual transformation in infertile patients

Due to the apparent alteration of the GR signalling pathway in ESCs isolated from infertile patients during decidual transformation in comparison to fertile patients, it was essential to investigate the effect of cortisol exposure on the expression of the previously chosen genes which are known to play a role in crucial processes within the human endometrium, particularly during the decidual response. There is also evidence that these genes i.e. WT1, FKBP4 and FKBP5 may be dysregulated within the endometrium of infertile pathologies.

Additionally, the effect of cAMP and F on the expression on AR (in PCOS patients) and PR (in endometriosis patients) was investigated.

In vitro decidualization in the absence and presence of cortisol resulted in no significant difference in the expression of AR at the protein level compared to control cells in ESCs isolated from both anPCOS and ovPCOS patients (figure 5.9A and B). AR is a key sex hormone receptor, which plays a critical role within the endometrium, where it is aberrantly expressed in PCOS patients. Additionally, treatment of these cells with cortisol only, resulted in no effect on AR protein expression in either patient groups studied. It was found that ovPCOS patients exhibited a higher expression of WT1, a gatekeeper of decidualization, compared to anPCOS patients, which is in agreement with previous work conducted by the RBGO group (figure 5.9A and C) (Gonzalez et al, 2012). It was found that *in vitro* decidualization of the ESCs isolated from PCOS patients resulted in decreases in the expression of WT1 compared to untreated control cells (significant only for anPCOS; $p \leq 0.05$). Co-treatment of the ESCs isolated from anPCOS patients with cAMP and F, resulted in a further significant decrease in the expression of WT1 compared to both control cells ($p \leq 0.01$) and cells treated with cAMP only ($p \leq 0.01$), whereas even though co-treatment with cAMP + F seems to down-regulate WT1 expression in the ESCs isolated from ovPCOS patients to some extent, no significant differences in endometrial WT1 expression were observed between any treatment group when compared to the control cells (figure 5.9A and C). Of further interest is that the down-regulation effect of WT1 when cells were treated with cAMP + F was maintained when cells were treated with F only and was significantly lower when compared to untreated cells ($p \leq 0.05$). Dissimilar results to that seen with WT1 were obtained when investigating the expression levels of FKBP5, where it was observed that co-treatment with cAMP and F and those treated with F alone, resulted in a significant increase in the expression levels of FKBP5 compared to untreated cells for anPCOS patients only ($p \leq 0.05$) (figure 5.9A and D). Even though co-treatment did not result in a significant increase in FKBP5 protein expression in ovPCOS patients compared to untreated control cells, it was however found that the co-treated cells resulted in an overall increase in FKBP5 protein expression compared to cells exposed to cAMP only ($p \leq 0.05$). This effect was also observed in ESCs isolated from anPCOS patients ($p \leq 0.01$). Finally, immunoblot analysis revealed that cAMP treatment did not result in any significant alterations in the expression of the prolactin protein regardless of treatment regime (figure 5.9A and E).

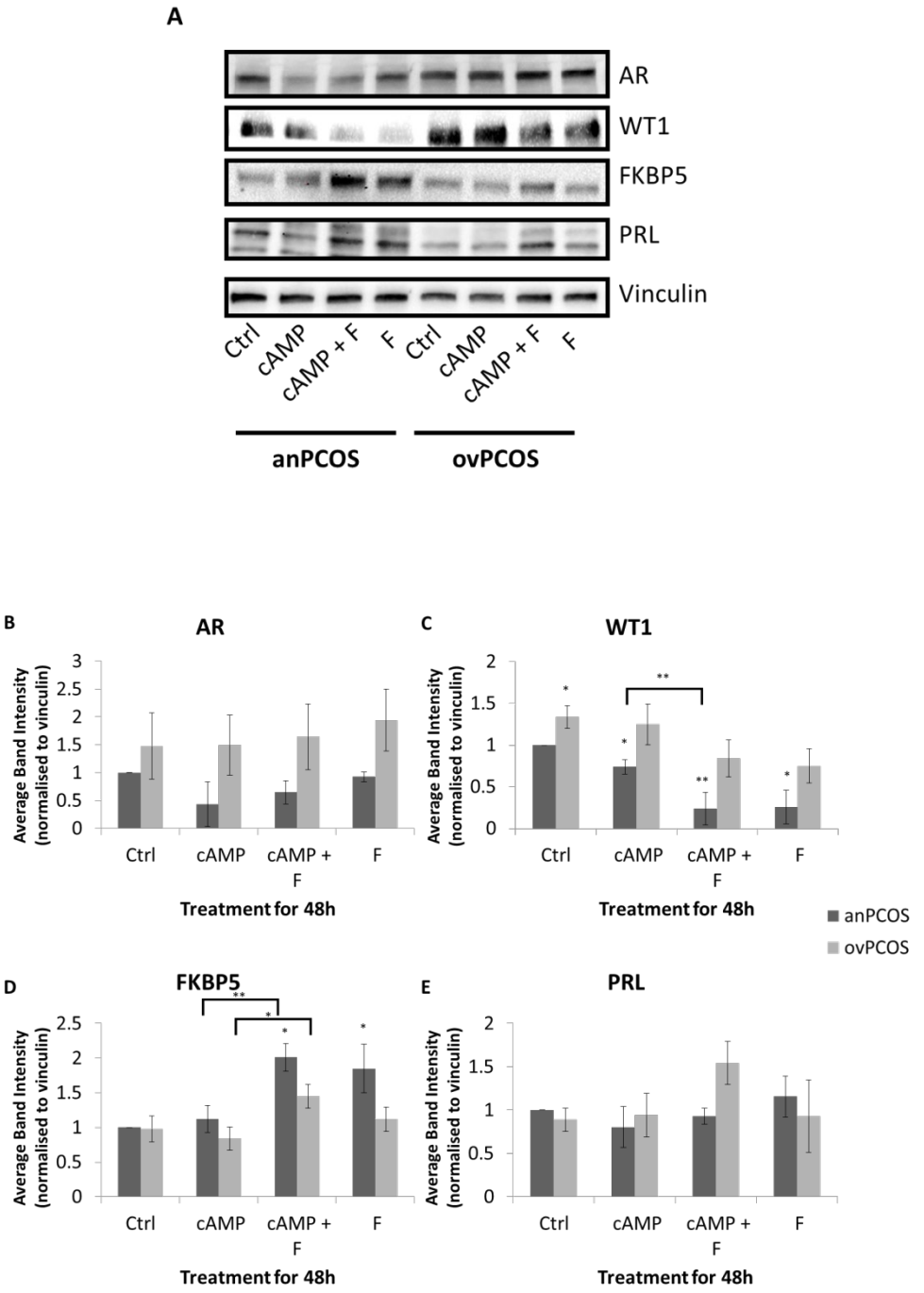


Figure 5.9 The effect of cortisol (F) on AR, WT1, FKBP5 and PRL during decidual transformation of ESCs isolated from PCOS patients (anovulatory and ovulatory). A) Representative images show the western blot analysis of AR (110 kDa), WT1 (52 kDa), FKBP5 (51 kDa), PRL (27 kDa) and vinculin (124 kDa) protein levels in ESCs isolated from PCOS patients (anPCOS: n=5 and ovPCOS: n=3) after 48h treatment with cAMP, cAMP and F or F alone and compared to the untreated control. B-C) The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the house keeper protein; vinculin. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

Immunoblot analysis was also carried out on ESCs isolated from endometriosis patients after 48h treatment with cAMP, cAMP and F or F only and compared to control cells.

In comparison, instead of investigating the effect of cAMP and F on the expression of AR, it was decided to instead investigate the expression of PR after treatment exposure. This is due to the significant impact PR has on the pathology of endometriosis. It was observed that *in vitro* decidualization did not have any effect on the expression of PR in ESCs isolated from endometriosis patients compared to untreated cells (figure 5.10A and B). However on addition of both cAMP and F, there was a significant decrease in the PR expression levels compared to both the control cells and cells treated with cAMP only ($p \leq 0.05$). This effect was also observed for WT1, where co-treatment with cAMP and F resulted in a significant reduction of WT1 protein levels compared to both the control cells and cells treated with cAMP only ($p \leq 0.05$) (figure 5.10 A and C). Likewise, co-treatment of the ESCs isolated from endometriosis patients also resulted in a significant decrease in the levels of PRL compared to untreated cells and those treated with cAMP ($p \leq 0.05$) (figure 5.10A and E). In contrast, the opposite effect was observed for FKBP5 protein levels, where co-treatment resulted in a significant induction in FKBP5 expression compared to the untreated cells and cells exposed to cAMP only ($p \leq 0.05$) (figure 5.10A and D). Treatment with cAMP only or F only resulted in no significant alterations in the expression of PR, WT1, FKBP5 or PRL at the protein level.

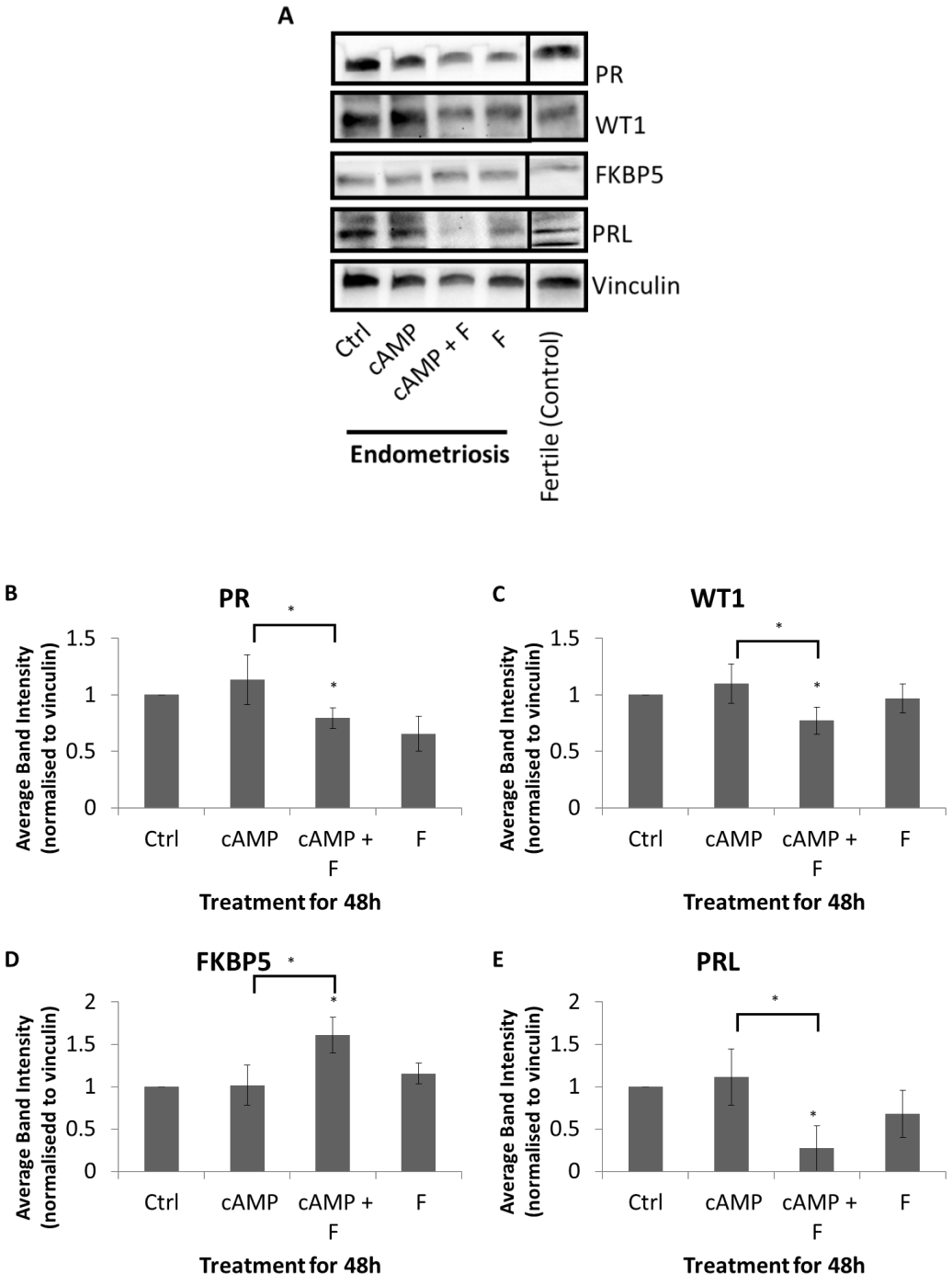


Figure 5.10 The effect of cortisol (F) on PR, WT1, FKBP5 and PRL during decidual transformation of ESCs isolated from endometriosis patients. A) Representative images show the western blot analysis of PR (110kDa), WT1 (52 kDa), FKBP5 (51 kDa), PRL (27 kDa) and vinculin (124 kDa) protein levels in ESCs isolated from endometriosis patients (n=6) after 48h treatment with cAMP, cAMP and F or F alone and compared to the untreated control. B-C) The intensity of the immunoblot bands was measured quantitatively using Image Lab software and normalised to the housekeeper protein; vinculin. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

5.2.3 The effect of cortisone during decidual transformation of endometrial stromal cells isolated from infertile patients

Following the investigation of the role of cortisol and the GR signalling pathway during decidual transformation of ESCs isolated from infertile patients, it was further decided to investigate the role of the biologically inactive glucocorticoid; cortisone, and therefore replicate the experiments carried out and described in section 4.2.3, which would allow for the comparison of fertile and infertile patients. This would further improve our understanding whether the infertile patients have any impairment of the GR signalling pathway and the interconversion of cortisol and cortisone.

Hence, to determine the role of cortisone (compound E) and the GR signalling pathway on decidual transformation of ESCs isolated from infertile patients, the ESCs were cultured and treated with the *in vitro* decidualization stimulus in combination with cortisone for 48h.

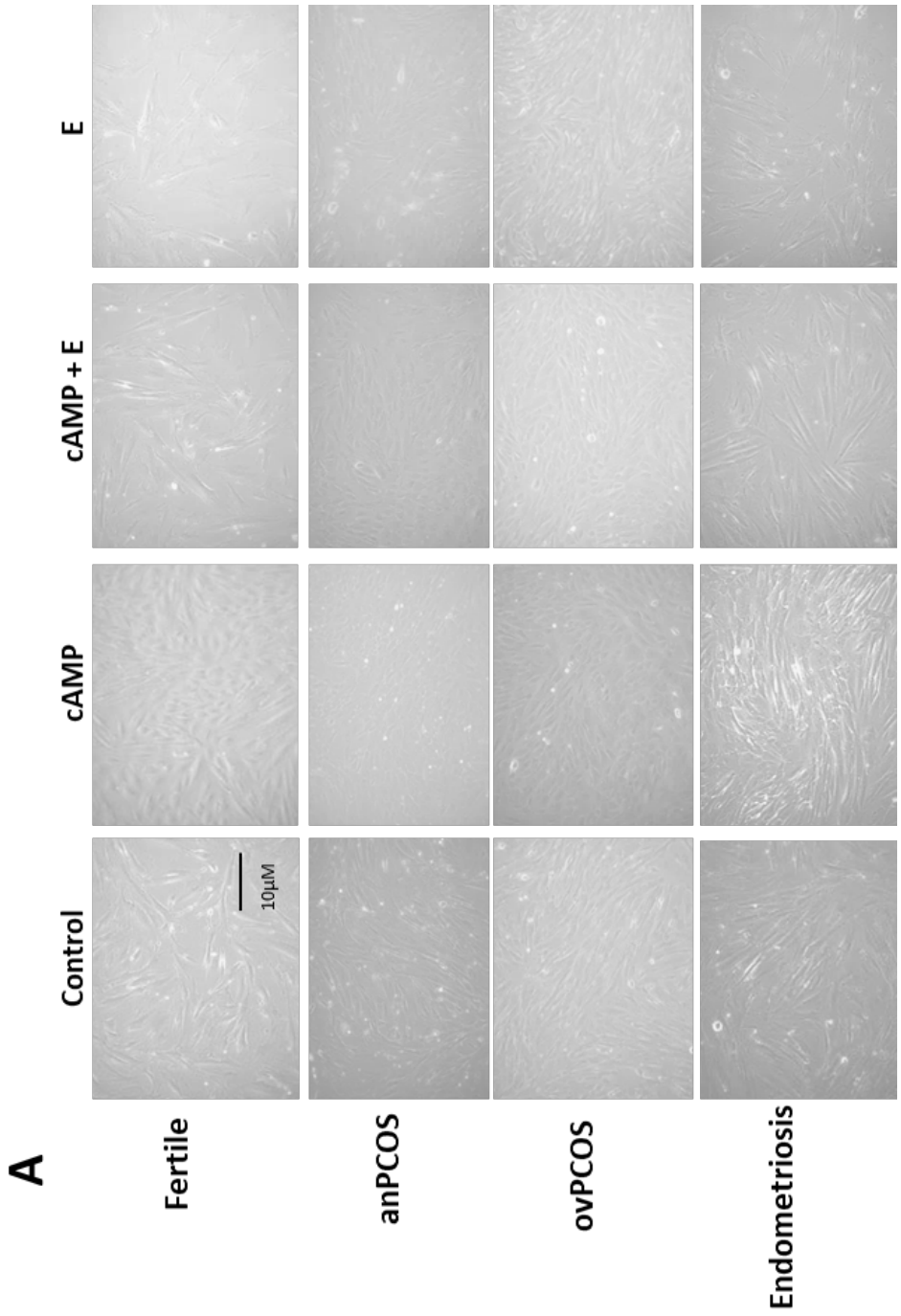
As previously described, decidualization was determined based on the morphological appearance of the ESCs and by the expression of key decidual markers.

5.2.3.1 The effect of cortisone on cell morphology and the expression of key decidual markers during decidual transformation in infertile patients

Cell monolayers were treated for 48h with cAMP, cAMP + E or E alone and compared to the untreated control cells. Results obtained after cell monolayers were treated with cAMP only have been previously described in section 5.2.2.1 and will not be described here.

As discussed in chapter 4 – the presence of the inactive hormone; cortisone also appears to impair successful decidualization taking place in the fertile patients in terms of cell morphology which is also demonstrated in figure 5.11A and B. Conversely, it was demonstrated that co-treatment with cAMP and E resulted in a significant increase in the average cell circularity values of ESCs isolated from anPCOS patients compared to untreated cells ($p \leq 0.05$) (figure 5.11). Additionally, co-treatment with cAMP and E appears to on average result in ESCs that have a more rounded phenotype compared to cells treated with cAMP only (not significant) where these alterations in morphology can be observed in the light microscopy images displayed in figure 5.11A, these results are similar to those obtained after cells were exposed to cAMP and F (section 5.2.2.1). Co-treatment with cAMP and E resulted in an increase in cell circularity values of ESCs isolated from patients with ovPCOS compared to untreated cells (figure 5.11), however, these values did not vary from the values obtained from cells treated with cAMP only, suggesting that the combination of

treatment of cAMP and E does not have any significant effect on the morphology of ESCs isolated from ovPCOS patients. On the other hand, ESCs isolated from endometriosis patients produced similar results to those obtained for fertile patients. It was found that co-treatment with cAMP and E appears to have an overall inhibitory effect on decidual transformation of the ESCs isolated from endometriosis patients compared to cells treated with cAMP only, where co-treatment resulted in comparable circularity values being obtained to the control cells (figure 5.11 A and B). Likewise, treatment with E alone resulted in no significant difference in the average circularity values obtained compared to untreated control cells in ESCs isolated from PCOS patients or endometriosis patients.



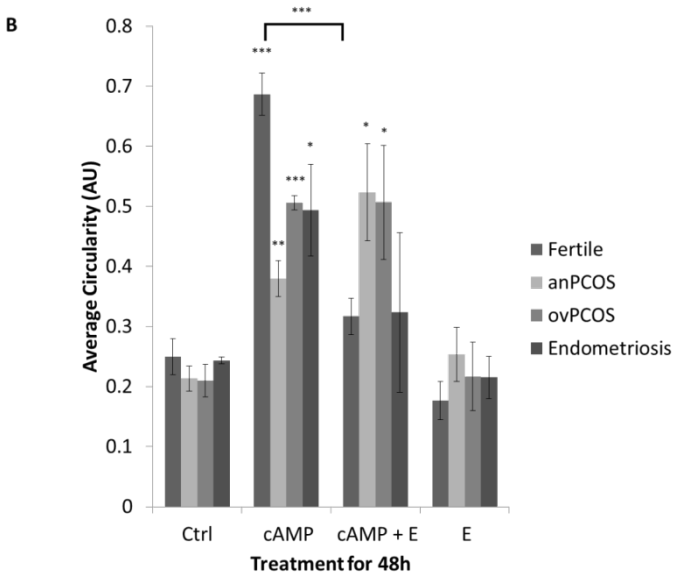


Figure 5.11A and 5.11B. The effect of cortisone (E) on the morphology and circularity values of ESCs isolated from infertile patients suffering from anPCOS, ovPCOS or endometriosis and compared to fertile patients during decidual transformation. A) Representative images taken of the cell monolayers after 48 h of treatments using inverted light microscopy (Zeiss) at an x10 objective. Scale bar indicates 10µM and applies to all images seen in this figure. These images were then used for Image J analysis to gain a quantitative measure of cell morphology. B) Thirty cells were chosen at random from each image taken of ESCs isolated from fertile patients (n=5), anPCOS patients (n=5), ovPCOS patients (n=3) and endometriosis patients (n=6). Values are averaged from 90 cells (HESCs) and 120 cells (ESCs isolated from fertile patients) and bars indicate ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

Additionally, to further support the morphological changes seen in these cells – the concentration of key secreted decidual markers were measured using commercially available ELISA kits.

The results obtained for cAMP treatment have been previously described and will therefore not be discussed here. It was found that treatment of the ESCs isolated from all infertile patients (PCOS and endometriosis patients) with cAMP and E resulted in a significant increase in the concentration of secreted prolactin compared to the untreated cells (p≤0.01) (figure 5.12A). However, even though this increase in secreted prolactin levels was observed, there was still a prominent difference in the protein levels compared to those obtained for the fertile patients who have the ability to undergo successful decidualization. Interestingly, co-treatment with cAMP and E resulted in a significant increase of the secretion of prolactin in ESCs isolated from anPCOS patients only compared to cells treated with the decidual stimulus only, whereas in all other patient groups, co-treatment appeared to on average result in a slight decrease in the secretion of prolactin into the culture media

compared to cells treated with cAMP only, but this was not deemed to be a statistically significant alteration (figure 5.12A).

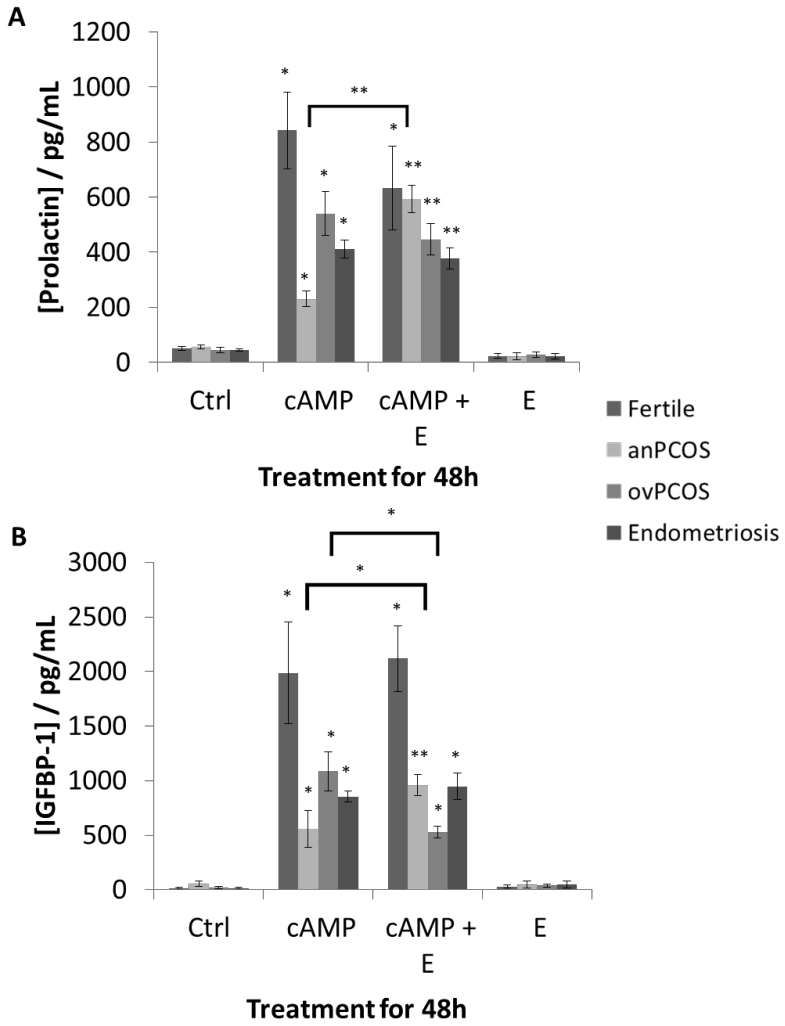


Figure 5.12. The effect of cortisone (E) on the levels of secreted decidual markers – prolactin (A) and IGFBP-1 (B) from ESCs isolated from infertile patients either suffering from anPCOS, ovPCOS or endometriosis and compared to fertile patients during decidual transformation. Media was extracted after ESCs isolated from fertile patients (n=5), anPCOS patients (n=5), ovPCOS patients (n=3) and endometriosis patients (n=6) underwent 48 h of treatment with cAMP, cAMP and E or E alone and compared to the control. ELISA experiments were carried out on the conditioned media to establish the concentration of key proteins at the secreted level. Treatment with cAMP caused a significant increase in the secreted levels of both decidual markers compared to the control in all pathology groups. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

Similar results were obtained for the secreted levels of IGFBP-1 (figure 5.12B). As seen with prolactin, treatment with ESCs isolated from infertile patients (PCOS and

endometriosis) with cAMP and E also resulted in a significant increase in the secreted levels of IGFBP-1 compared to untreated cells. It was also found that ESCs isolated from anPCOS patients resulted in significantly higher levels of IGFPBP-1 secretion compared to cells treated with cAMP only ($p \leq 0.05$) (figure 5.12B). Moreover, ESCs isolated from ovPCOS patients treated with cAMP and E, resulted in an increase in the secreted concentration of IGFBP-1 compared to cAMP treatment only ($p \leq 0.05$). Conversely, co-treatment of cells isolated from either fertile or patients suffering from endometriosis does not seem to have any effect on IGFBP-1 secretion compared to untreated control cells or cells treated with the decidual stimulus only (figure 5.12B). It is important to note that even though there is a statistically significant induction of the expression and secretion of IGFBP-1 in the infertile patients, the concentrations obtained are significantly lower than those seen in ESCs isolated from fertile patients. This further indicates that decidualization is impaired to varying extents in these patient cohorts.

These ELISA results were further confirmed using qPCR to determine the expression levels of dPRL and IGFBP-1 at the mRNA level using gene specific primers.

Analysis of the results indicated that both dPRL and IGFBP-1 expression was significantly induced in all patient groups when co-treated with cAMP and E to varying extents ($p \leq 0.05$) (figure 5.13A and B), therefore supporting the ELISA results described previously. However, using qPCR analysis there were no differences in the expression of either dPRL or IGFBP-1 at the mRNA level in ESCs isolated from anPCOS or ovPCOS patients treated with cAMP and E compared to those treated with cAMP only. It was found in all patient groups that co-treatment with cAMP and E did not result in any significant alterations in the gene expression levels of the decidual markers, even though overall differences in expression levels were observed. For example, cAMP and E treatment seems to result in an average increase in dPRL mRNA expression levels in anPCOS patients compared to cAMP treatment only and additionally, cAMP and E treatment seems to result in an on average decrease in IGFBP-1 mRNA expression levels in ovPCOS patients compared to cAMP treatment only. However due to variations seen using qPCR analysis, none of these effects are significant. This therefore suggests that cAMP and E treatment may only be significantly altering the secreted levels of the decidual proteins in the ESCs isolated from the PCOS patients.

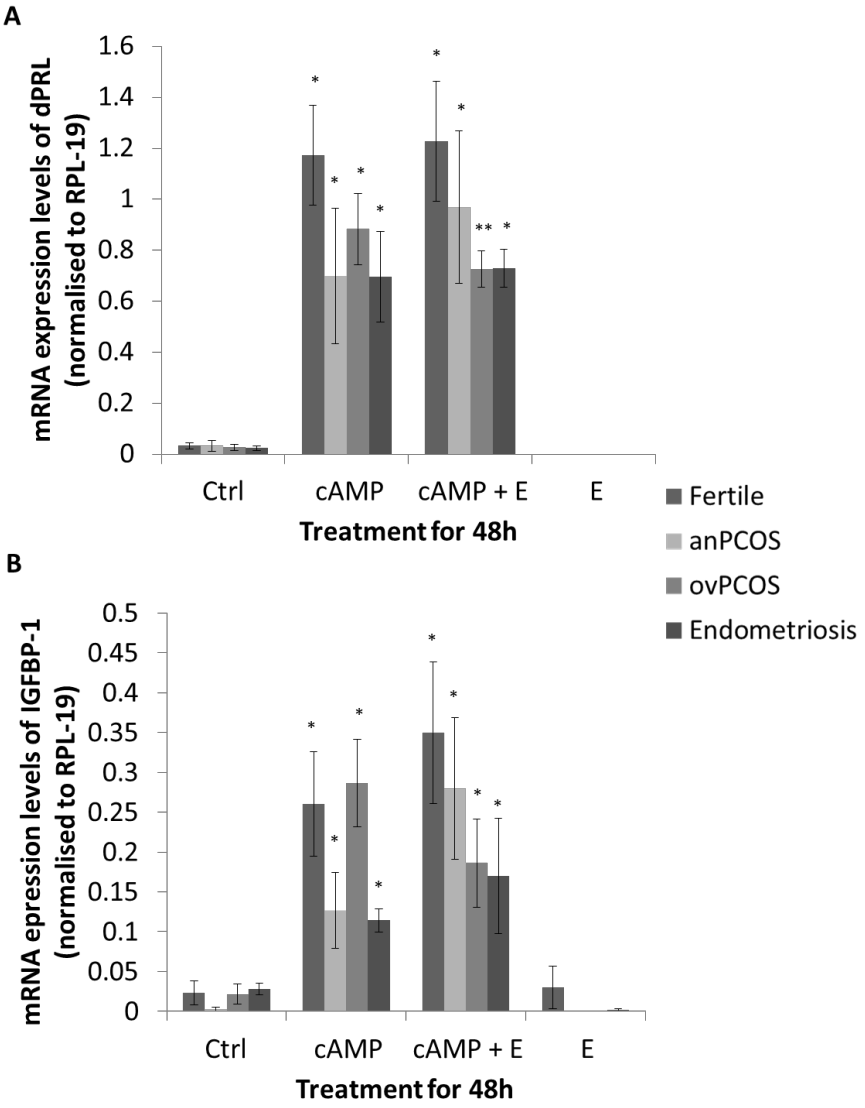


Figure 5.13 The effect of cortisone (E) on the levels of the mRNA expression levels of key decidual markers – decidual prolactin (dPRL) (A) and IGFBP-1 (B) in ESCs isolated from infertile patients suffering from either anPCOS, ovPCOS or endometriosis and compared to fertile patients during decidual transformation. After 48 h of treatment, ESCs isolated from fertile patients (n=5), anPCOS patients (n=5), ovPCOS patients (n=3) and endometriosis patients (n=6) were lysed, RNA extracted and converted to cDNA and used for qPCR analysis. Cells underwent 48 h of treatment with cAMP, cAMP and E or E alone and compared to the control. qPCR analysis was carried out to establish the expression levels at the mRNA level of dPRL (A) and IGFBP-1 (B). Treatment with cAMP alone and cAMP and E, caused an increase in the expression levels of both decidual markers compared to the control. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

5.2.3.2 The effect of cortisone on the expression of GR and 11β-HSD during decidual transformation in infertile patients

To test whether exposure to cAMP and E was differentially affecting the GR signalling pathway in the ESCs isolated from infertile patients, the expression of GR and the 11β-HSD enzymes during decidual transformation, both in the absence and presence of cortisone was investigated. As previously described, the ESCs isolated from infertile patients were cultured and treated with cAMP, cAMP + E or E alone for 48h and compared to the untreated control cells.

Results obtained for *in vitro* decidualization of ESCs isolated from anPCOS, ovPCOS and endometriosis patients have been discussed previously in this chapter and will not be discussed here. It was found that in vitro decidualization of ESCs isolated from anPCOS patients in the presence of E had no significant effect on the protein expression of GRα or GRβ compared to untreated control cells (figure 5.14A, B and C). However, a significant increase in the protein expression levels of GRα were observed in ESCs isolated from ovPCOS patients co-treated with cAMP and E compared to the control ($p \leq 0.05$), similar to the levels seen when these cells were treated with cAMP alone (figure 5.14A and B). Conversely, co-treatment with cAMP and E had no significant effect on the protein levels of GRβ (figure 5.14A and C). Additionally, treatment of ESCs isolated from both anPCOS and ovPCOS patients with E alone had no significant effect on the expression of either GR isoform.

Immunoblot analysis was also carried out to investigate the effect of E on the expression of the 11β-HSD enzymes during *in vitro* decidualization. It was found that co-treatment with cAMP and E had no significant effect on the expression of 11β-HSD1 enzyme compared to the control cells and cells treated with cAMP only in both the anPCOS and ovPCOS patient groups (figure 5.14A and D). However, analysis revealed that co-treatment of ESCs isolated from anPCOS patients only with cAMP and E resulted in a significant increase in the protein expression of 11β-HSD2 compared to untreated ESCs (figure 5.14A and E). Similar to results obtained for GR, treatment with E alone did not result in any significant alterations in 11β-HSD protein expression in either anPCOS or ovPCOS patient cells.

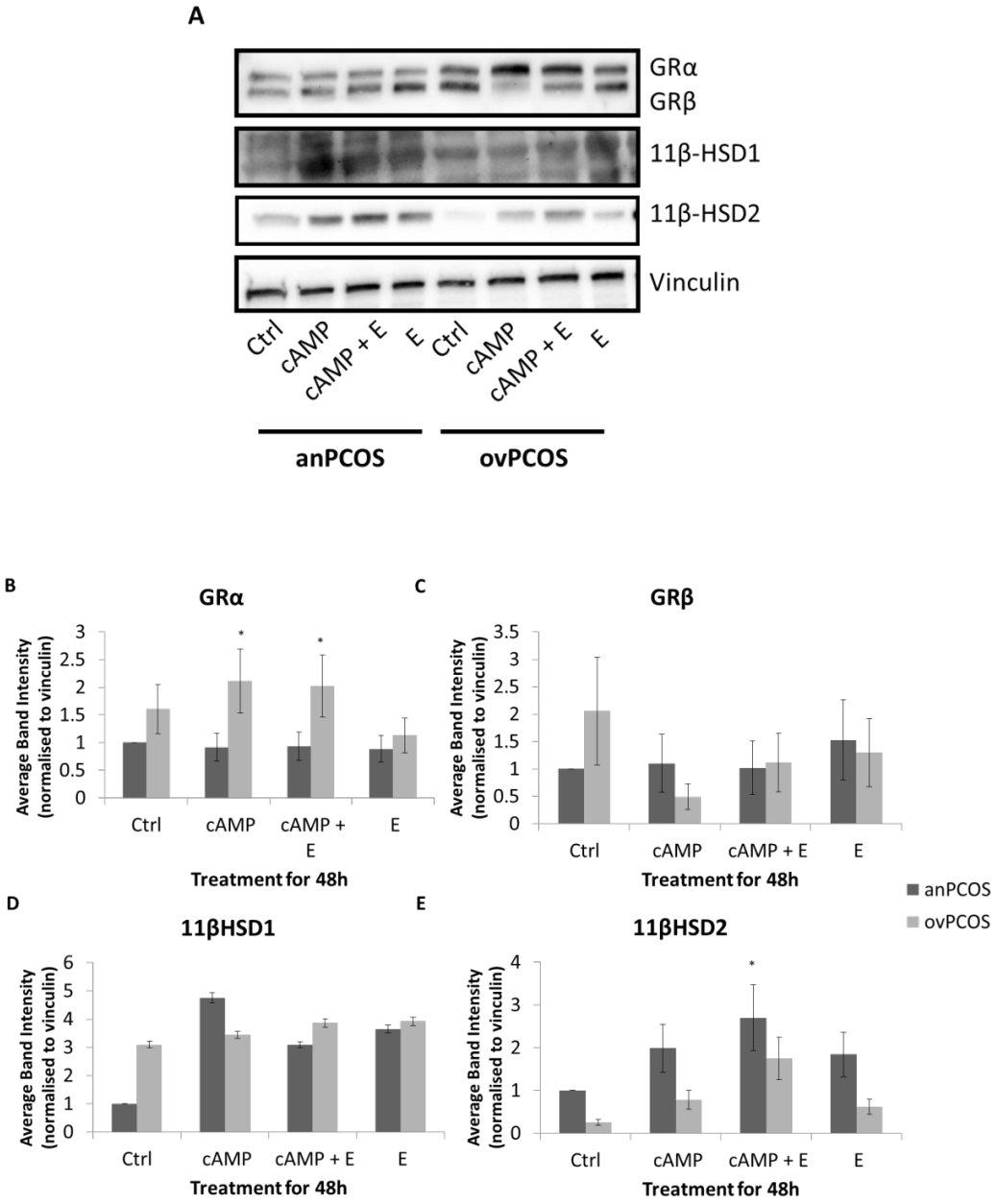


Figure 5.14 The effect of cortisone (E) on key proteins involved in the GR signalling pathway during decidual transformation in ESCs isolated from infertile patients either suffering from anPCOS or ovPCOS. A) Representative images show the western blot analysis of GRα (95kDa), GRβ (90kDa), 11βHSD1 (32 kDa) and 11βHSD2 (40kDa) protein levels in ESCs isolated from anPCOS patients (n=5) vs ESCs isolated from ovPCOS patients (n=3) after 48h treatment with cAMP, cAMP and E or E alone and compared to the untreated control. B-E) The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the house keeper protein; vinculin (124 kDa). Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

Immunoblot analysis was also carried out on ESCs isolated from endometriosis patients. As mentioned for results described in figure 5.14, results obtained for cAMP treatment only have been described previously in this chapter and will not be discussed here. It was found that co-treatment of ESCs isolated from endometriosis patients with cAMP and E resulted in no alterations of GRα expression compared to untreated cells (figure 5.15A and B). However, co-treatment did result in a decrease in the levels of GRβ at the protein level compared to both untreated control cells and cells treated with cAMP only ($p \leq 0.05$ and $p \leq 0.05$) (figure 5.15A and C). Furthermore, treatment with E alone also resulted in a significant decrease in GRβ protein expression in ESCs isolated from endometriosis patients compared to untreated cells ($p \leq 0.05$) (figure 5.15A and C).

In addition to alterations in GR expression levels, it was found that in vitro decidualization of the ESCs isolated from endometriosis patients in combination with E treatment resulted in no statistically significant changes in the expression of 11β-HSD1 at the protein level compared to the untreated cells, however on average there is an overall decrease in 11β-HSD1 expression levels compared to cells treated with cAMP only which was not statistically significant (figure 5.15A and D). Contrariwise, co-treatment resulted in an increase in the protein expression levels of 11β-HSD2 enzyme compared to both untreated cells and cells treated with cAMP only, but again this was not deemed statistically significant (figure 5.15A and E). ESCs treated with E only, resulted in a decrease in the expression levels of 11β-HSD1 (not significant) but had no effect on the expression of 11β-HSD2 protein expression.

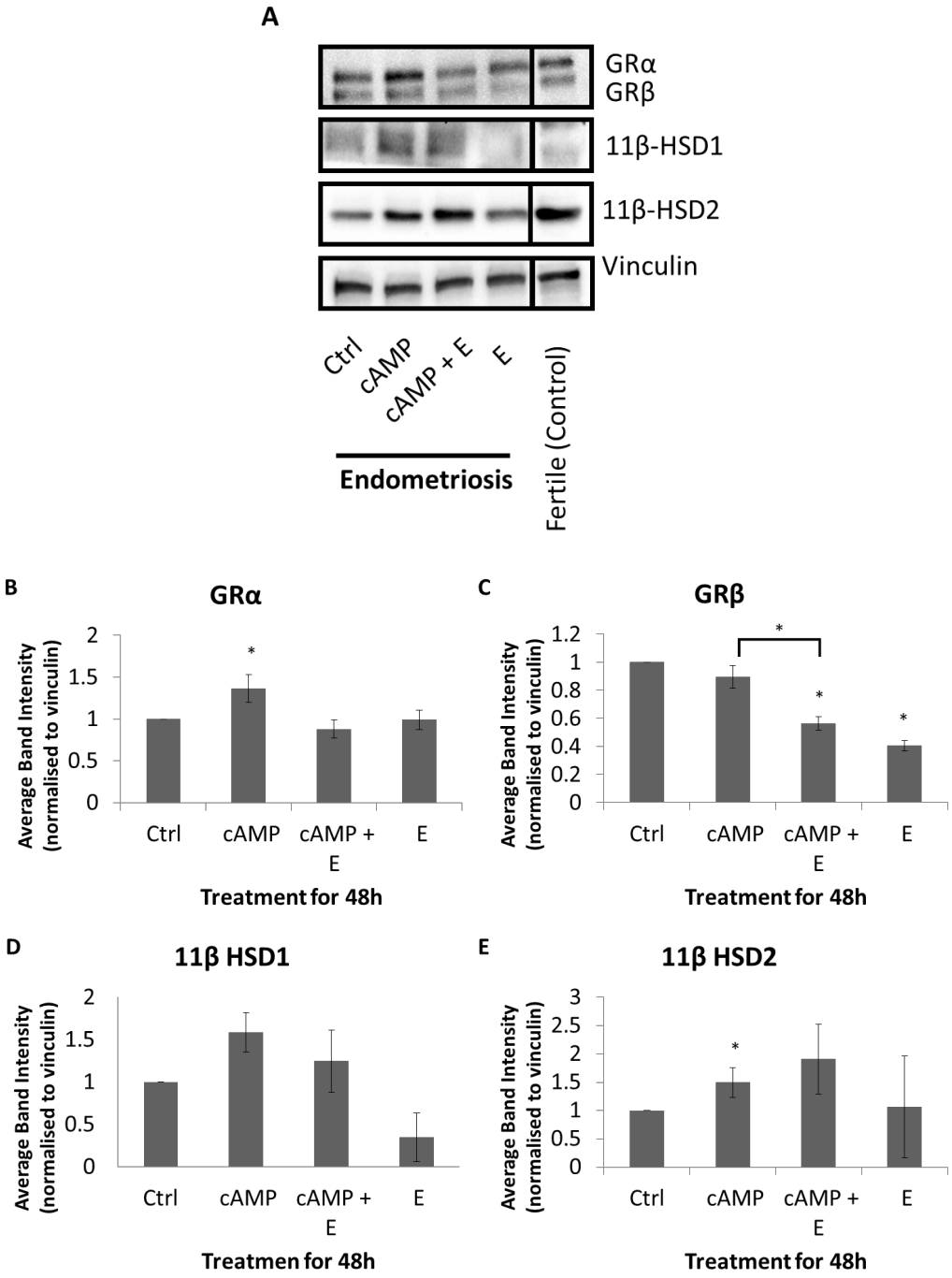


Figure 5.15 The effect of cortisone (E) on key proteins involved in the GR signalling pathway during decidual transformation in ESCs isolated from infertile patients suffering from endometriosis. A) Representative images show the western blot analysis of GRα (95kDa), GRβ (90kDa), 11βHSD1 (32 kDa) and 11βHSD2 (40 kDa) protein levels in ESCs isolated from endometriosis patients (n=6) after 48h treatment with cAMP, cAMP and E or E alone and compared to the untreated control. Untreated ESCs isolated from a fertile patient was also included as a reference for basal levels. B-E) The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the house keeper protein; vinculin (124kDa). Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

5.2.3.3 The effect of cortisone on the expression of potential GR targets during decidual transformation in infertile patients

In addition to cortisol, it was also decided to investigate whether exposure to cortisone could alter the expression of a selection of genes which are known to be essential within the human endometrium.

Western blot analysis revealed that *in vitro* decidualization in the presence and absence of E, resulted in a significant decrease in the expression of WT1 in ESCs isolated from anPCOS patients (figure 5.16A and B) at the protein level compared to untreated cells ($p \leq 0.05$). Whereas, treatment of ESCs isolated from ovPCOS patients with cAMP and E had no significant effect on the protein expression levels of WT1 compared to untreated cells. Co-treatment of ESCs isolated from anPCOS and ovPCOS patients with cAMP + E, resulted in a significant increase in FKBP5 protein expression compared to untreated cells ($p \leq 0.01$ and $p \leq 0.05$ respectively). In ESCs isolated from ovPCOS patients only there was a significant induction in FKBP5 expression compared to cells treated with cAMP only ($p \leq 0.05$) (figure 5.16A and C). Interestingly, treatment with E alone, also resulted in a significant increase in the expression of FKBP5 at the protein level in both ESCs isolated from anPCOS and ovPCOS patients ($p \leq 0.01$ and $p \leq 0.05$ respectively) (figure 5.16 A and C). Finally, immunoblot analysis revealed that treatment of ESCs isolated from either anPCOS or ovPCOS patients with cAMP and E or E alone did not result in any significant alterations in the expression of PRL at the protein level (figure 5.16 A and D). However densitometry band analysis of the immunoblots did reveal that there was an overall inhibition of endometrial PRL expression in ESCs isolated from ovPCOS patients treated with cAMP + E or E alone compared to untreated control cells and cells treated with cAMP only.

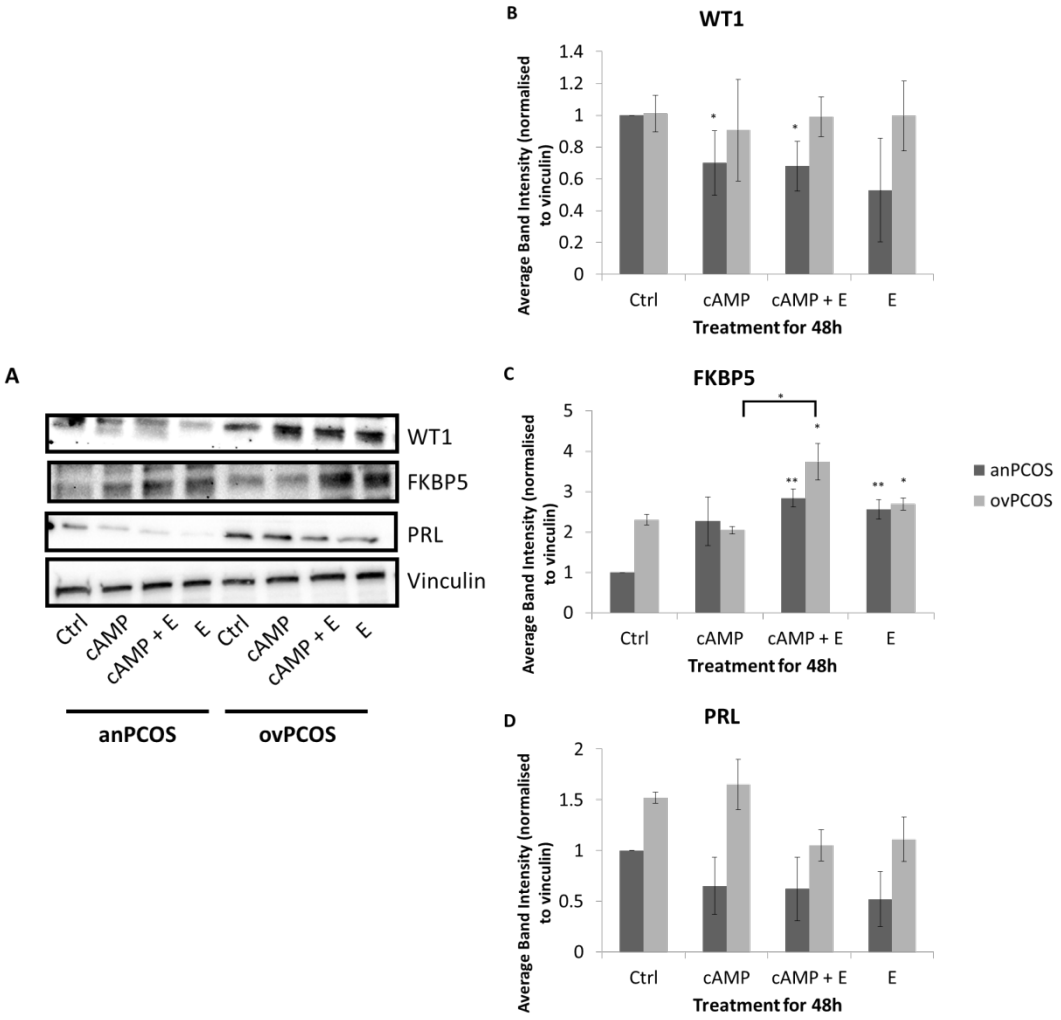


Figure 5.16 The effect of cortisone (E) on WT1, FKBP5 and PRL during decidual transformation of ESCs isolated from PCOS patients (anovulatory and ovulatory). A) Representative images show the western blot analysis of WT1 (52kDa), FKBP5 (51kDa), PRL (27 kDa) and vinculin (124 kDa) protein levels in ESCs isolated from PCOS patients (anPCOS: n=5 and ovPCOS: n=3) after 48h treatment with cAMP, cAMP and E or E alone and compared to the untreated control. B-D) The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the house keeper protein; vinculin. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

Immunoblot analysis was also carried out on ESCs isolated from endometriosis patients after 48h treatment with cAMP, cAMP and E or E alone and controlled to untreated control cells.

It was observed that ESCs co-treated with cAMP and E had no significant effect on the WT1 protein expression compared to untreated cells (figure 5.17A and B). However, immunoblot analysis reveals an overall decrease in WT1 expression compared to cells treated with cAMP only, but this was found to be non-significant (figure 5.17B). Conversely, co-treatment with cAMP and E resulted in a significant decrease in the expression of both FKBP5 (figure 5.17

A and C) and PRL (figure 5.17A and D) compared to untreated cells ($p \leq 0.05$ and $p \leq 0.01$ respectively). On visual inspection of the immunoblots, it appears that they are co-treated ESCs isolated from endometriosis patients also have a lower expression of FKBP5 and PRL compared to cells treated with cAMP only, but again this was not found to be statistically significant. Treatment with E alone resulted in significant decreases in the expression of WT1, FKBP5 and PRL at the protein level when compared to the expression levels seen in untreated cells ($p \leq 0.05$) (figure 5.17A-D).

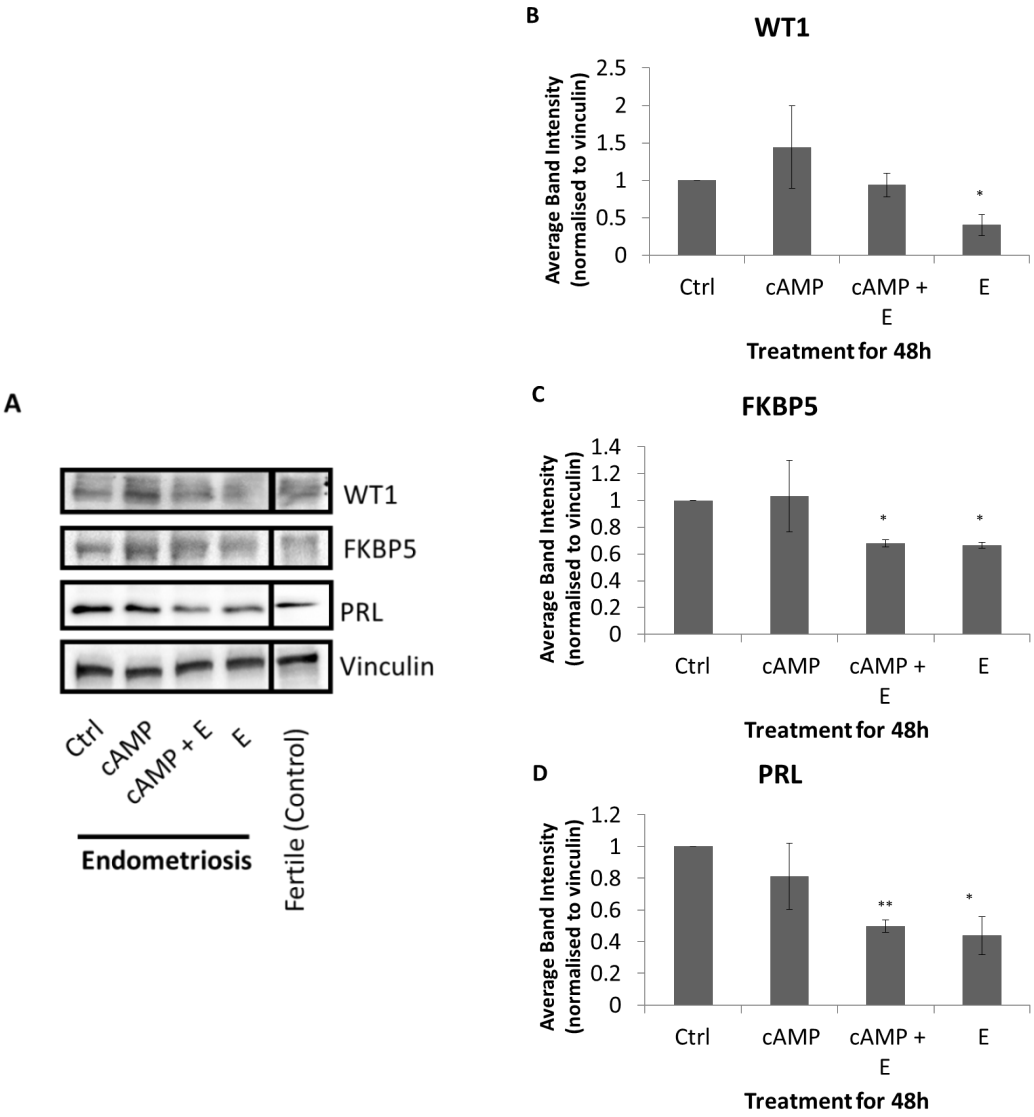


Figure 5.17 The effect of cortisone (E) on WT1, FKBP5 and PRL during decidual transformation of ESCs isolated from endometriosis patients. A) Representative images show the western blot analysis of WT1 (52 kDa), FKBP5 (51 kDa), PRL (27 kDa) and vinculin (124 kDa) protein levels in ESCs isolated from endometriosis patients (n=6) after 48h treatment with cAMP, cAMP and E or E alone and compared to the untreated control. B-D) The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the housekeeper protein; vinculin. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ are considered significant.

5.2.4 The effect of androgens and the androgen receptor (AR) on the GR signalling pathway in infertile patients

Due to the results obtained previously, it was decided to investigate whether the aberrant expression of AR seen within the endometrium of PCOS patients has any effect on the GR signalling pathway. Additionally, it was of interest to determine whether the high circulating levels of androgens present in these patients that ultimately give rise to a wide range of the clinical manifestations of this syndrome also may be playing a role in the HPA axis and stress response within these patients. Additionally, as previous research suggests there may be potential crosstalk between GR and the sex steroid hormone receptors, including AR.

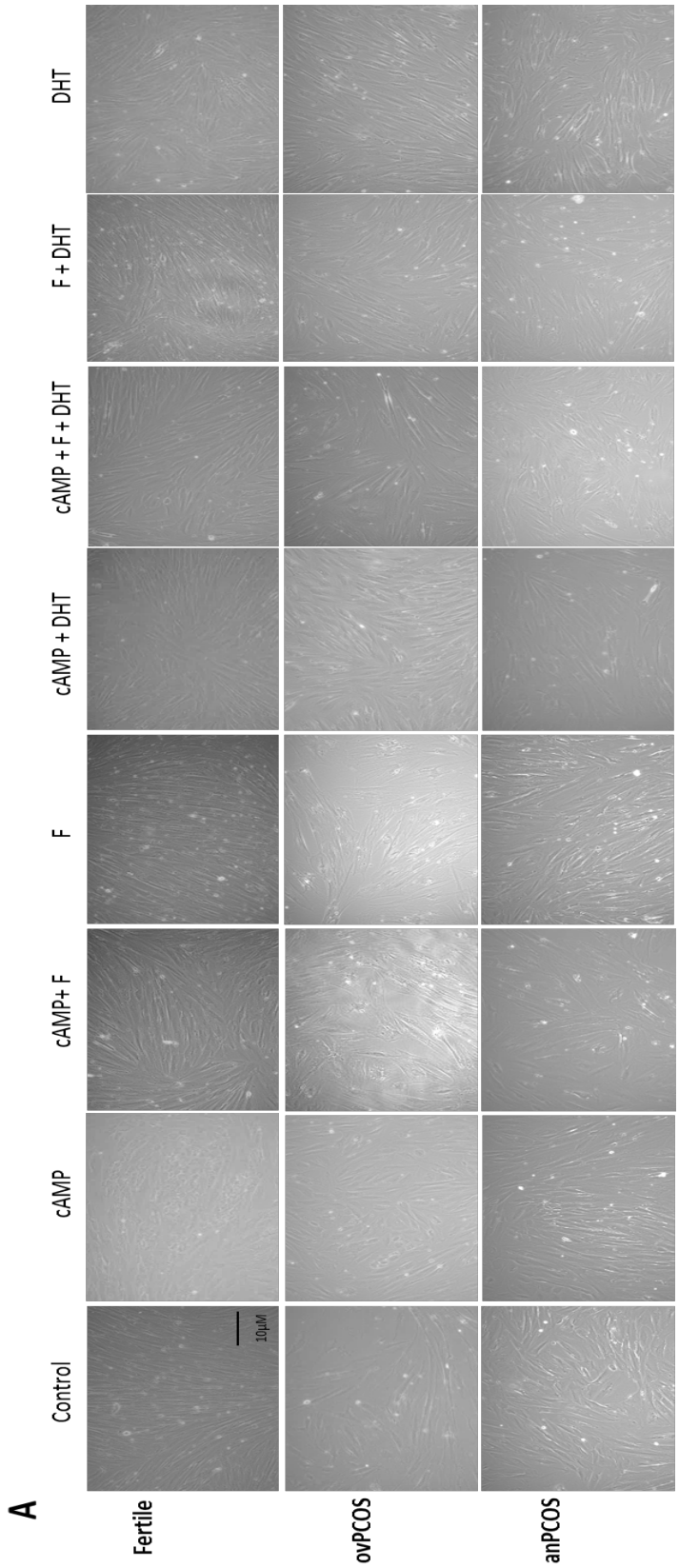
Therefore, initial experiments were conducted to identify whether the presence of both the stress hormone cortisol and excess androgens, has any effect on the *in vitro* decidual response of these cells and key proteins involved in the GR signalling pathway.

5.2.4.1 The effect of cortisol and androgens on cell morphology and the expression of key decidual markers during decidual transformation

Cell monolayers were treated for 48h with either cAMP + DHT, cAMP + F + DHT, F + DHT or DHT only and compared to the previously described cells, which had been treated with cAMP only, cAMP + F and F alone. Untreated control cells were also included in these experiments. Results have been previously described for ESCs isolated from fertile and infertile PCOS patients treated with cAMP, cAMP + F and F alone and therefore will not be described in detail here but are included to ensure a direct comparison can be made.

It was found that treatment with the *in vitro* decidualization stimulus cAMP and DHT resulted in a visual change of the cell morphology from an elongated fibroblast-like shape to a more rounded epithelial-like phenotype (figure 5.18A). This alteration of cell morphology was confirmed quantitatively using Image J software. It was found that ESCs isolated from fertile, ovPCOS and anPCOS patients treated with cAMP + DHT resulted in a significant increase in cell circularity compared to their corresponding untreated cells ($p \leq 0.01$, $p \leq 0.05$ and $p \leq 0.05$ respectively) (figure 5.18B). However, it was found that fertile ESCs co-treated with cAMP + DHT resulted in significantly lower average circularity value being obtained compared to cells treated with cAMP only ($p \leq 0.01$). Furthermore, treatment of ESCs isolated from fertile, ovPCOS and anPCOS patients treated with cAMP, F and DHT also resulted in a significant increase in average cell circularity values compared to the untreated cells ($p \leq 0.05$ for all), which can also be seen visually in the inverted light microscopy

images (figure 5.18 B and A respectively). However, the addition of F treatment to the ESCs isolated from fertile patients, resulted in a significant decrease in the average cell circularity values compared to those treated with cAMP and DHT only ($p \leq 0.05$).



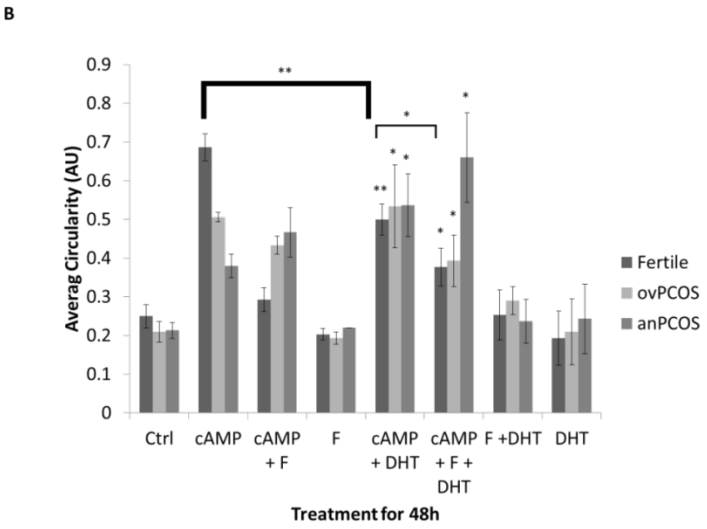


Figure 5.18A and 5.18B. The effect of cortisol (F) and androgen exposure on the morphology and circularity values of ESCs isolated from infertile patients suffering from either anPCOS or ovPCOS and compared to fertile patients during decidual transformation. A) Representative images taken of the cell monolayers after 48 h of treatments using inverted light microscopy (Zeiss) at an x10 objective. Scale bar indicates 10μM and applies to all images seen in this figure. These images were then used for Image J analysis to gain a quantitative measure of cell morphology. B) Thirty cells were chosen at random from each image taken of ESCs isolated from fertile patients (n=5), anPCOS patients (n=5) and ovPCOS patients (n=3). Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

Looking at the multiple images that were taken of the cell monolayers, it appeared that the addition of F to the cAMP and DHT treated ESCs isolated from anPCOS was enhancing the number of cells adopting a more rounded phenotype, but quantitative analysis revealed that even though on average there was an increase in the average circularity values – this was not statistically significant. Finally, treatment of all three patient groups with either F and DHT or DHT alone resulted in no significant variation of cell circularity value compared to the untreated ESCs (figure 5.18A and B).

To further support the morphological changes seen in these cells – the concentration of key secreted decidual markers was measured using commercially available ELISA kits. The results obtained from the ELISA supported the cell morphology results presented in figure 5.18.

It was found that treatment of the ESCs isolated from fertile, anPCOS and ovPCOS patients with cAMP + DHT, resulted in a significant increase in the concentration of secreted prolactin compared to their corresponding untreated cells (all p≤0.05) (figure 5.19A). Additionally, for anPCOS patients only, it was found that co-treatment with cAMP and DHT

also resulted in a significant increase in the concentration of prolactin present compared to ESCs exposed to the decidualization stimulus only ($p \leq 0.05$), which was not reciprocated in cells isolated from fertile or ovPCOS patients. Interestingly, treatment of all three patient groups with cAMP, DHT and F also resulted in a significant increase in the concentration of prolactin present (all $p \leq 0.05$). It is important to note that on average it seemed that the addition of F as a treatment to the ESCs isolated from fertile patients in addition to cAMP and DHT, seemed to result in a decreased in the levels of secreted prolactin, but this was not statistically significant (figure 5.19A).

Comparable results were obtained for secreted levels of IGFBP-1 (figure 5.19B). Similar to PRL, it was found that co-treatment of ESCs isolated from fertile, ovPCOS and anPCOS patients with cAMP and DHT resulted in a significant increase in the secretion levels of IGFBP-1 compared to untreated cells ($p \leq 0.05$. $p \leq 0.05$ and $p \leq 0.01$ respectively). As was the case for PRL, treatment of ESCs isolated from anPCOS patients with cAMP and DHT also resulted in a significant increase of IGFBP-1 secretion compared to cells treated with cAMP only ($p \leq 0.05$). Furthermore, interesting results were obtained for the cAMP, F and DHT treatment groups, where it was found that this co-treatment resulted in a significant increase in the levels of secreted IGFBP-1 in ESCs isolated from fertile, ovPCOS and anPCOS patients ($p \leq 05$, $p \leq 0.01$ and $p \leq 0.01$ respectively) compared to untreated cells. In the fertile patients only, there was also a significant increase in IGFBP-1 concentration levels compared to cells treated with cAMP and F only ($p \leq 0.05$) (figure 5.19B). Treatment with cAMP, F and DHT had no significant effect on the IGFBP-1 secreted levels in either cohort of PCOS patients compared to cells treated with cAMP and F only. For both PRL and IGFBP-1, treatment with either F and DHT, F only or DHT only resulted in no alterations in the secreted levels compared to untreated cells.

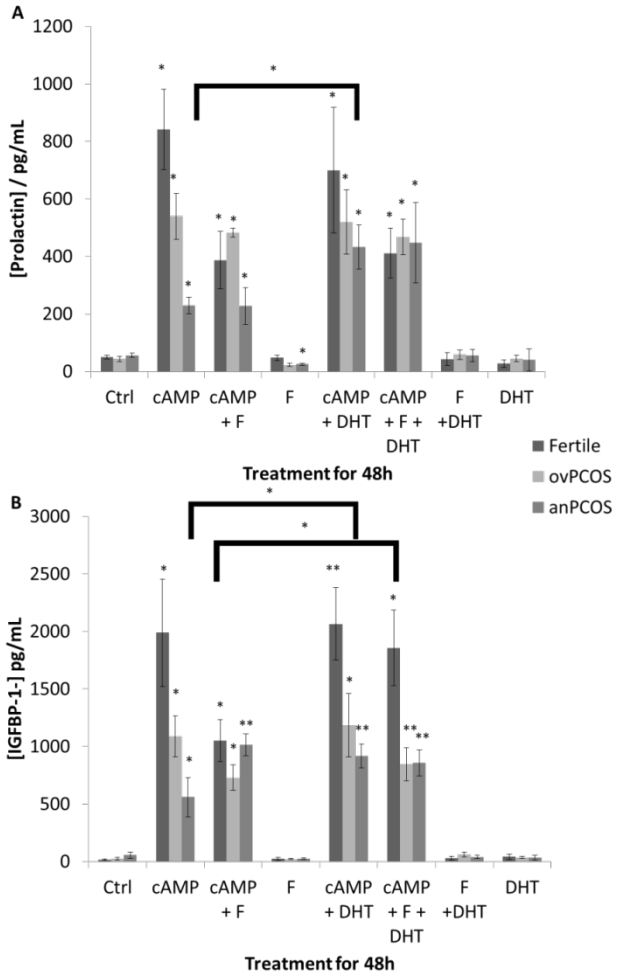


Figure 5.19. The effect of cortisol (F) and androgen exposure on the levels of secreted decidual markers – prolactin (A) and IGFBP-1 (B) from ESCs isolated from infertile patients either suffering from anPCOS or ovPCOS and compared to fertile patients during decidual transformation. Media was extracted after ESCs isolated from fertile patients (n=5), anPCOS patients (n=5) and ovPCOS patients (n=3) and underwent 48 h of treatment with either cAMP, cAMP and F, F alone, cAMP and DHT, cAMP and F and DHT, F and DHT or DHT alone and compared to their corresponding untreated cells. ELISA experiments were carried out on the conditioned media to establish the concentration of key proteins at the secreted level. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

5.2.4.2 The effect of cortisol and androgens on the expression of GR, AR and 11β-HSD during decidual transformation

To further investigate the interaction between the GR and AR signalling pathways, it was decided to investigate to effect of cortisol and androgen exposure on the expression of key proteins involved in the GR signalling pathway in addition to AR. Previous results in this chapter, suggest that there may be a cumulative effect of cortisol and the androgen; DHT, in terms of its effect on a cell's availability to undergo decidualization. Therefore, it was of

interest to elucidate the effect of these hormones on the sex steroid hormone signalling pathways.

Experiments were first conducted on ESCs isolated from fertile patients. Immunoblot analysis revealed interesting results, where it was shown that treatment of fertile ESCs wither either cAMP + DHT, cAMP + F + DHT, F + DHT or DHT alone resulted in a significant increase in the protein expression levels of AR ($p \leq 0.001$. $p \leq 0.01$, $p \leq 0.001$ and $p \leq 0.001$ respectively) (figure 5.20A and B). The addition of DHT during decidual transformation also resulted in a significant increase in AR expression compared to cells treated with cAMP only ($p \leq 0.01$). This effect was present either in the absence of presence of F, where treatment with cAMP, F and DHT also resulted in a significant increase in AR expression compared to cells treated with cAMP + F only. These results indicate that the presence of DHT results in a significant induction of AR protein expression, regardless of the presence of the stress hormone or the decidualization stimulus. On the immunoblot analysis, protein extracted from ESCs isolated from anPCOS patients was also included for a direct comparison to the fertile patients (figure 5.20 A and B). Where it can be seen that ESC isolated from anPCOS patients have a significantly higher expression level of AR compared to ESCs isolated from fertile patients ($p \leq 0.01$).

Immunoblots were also carried out to determine the expression of the two GR isoforms in the fertile patients (figure 5.20A, C and D). It was found that co-treatment of ESCs isolated from fertile patients with cAMP and DHT resulted in a significant induction of GRα protein expression compared to untreated cells ($p \leq 0.05$) (figure 5.20A and C). Whereas on the addition of F treatment, this effect was overall reduced compared to cells treated with cAMP and DHT only (not significant). Furthermore, treatment with DHT only resulted in a similar level of induction of GRα expression to that seen in the cAMP and DHT treatment group, which was significantly higher than the untreated cells ($p \leq 0.05$). Treatment of the ESCs isolated from fertile patients with either F + DHT or F only resulted in a significant reduction of GRα expression compared to untreated cells ($p \leq 0.05$) (figure 5.20A and C). Finally, as previously mentioned in this chapter – ESCs isolated from anPCOS patients have a significantly higher expression of GRα compared to fertile patients ($p \leq 0.05$). In addition to GRα, immunoblots were also used to study the protein expression levels of GRβ. Conversely, it was found that treatment of ESC isolated from fertile patients with cAMP, F and DHT, F and DHT or F only resulted in a significant reduction of GRβ expression at the protein level ($p \leq 0.05$ for all) (figure 5.20A and D). Treatment with cAMP and DHT and also cAMP and F also resulted in an overall reduction in GRβ expression, but this was not

significant. ESCs isolated from anPCOS patients exhibit a lower expression of GRβ at the basal level compared to fertile patients (figure 5.20A and D).

In addition to GR, the enzymes which are responsible for the bioavailability of the active ligand of GR were also investigated. Results were varied, but overall it appeared that co-treatment of ESCs isolated from fertile patients with a combination of cAMP, F and DHT resulted in an increase in 11β-HSD1 expression at the protein level compared to untreated cells ($p \leq 0.05$) (figure 5.20 A and E). Additionally, treatment with DHT only also resulted in a significant induction of 11β-HSD1 expression compared to untreated cells. It is important to note that some slight changes in expression were also observed for other treatment groups, but these changes were not deemed to be statistically significant when an average was taken for the values obtained from the group of patients.

As witnessed previously in this study, it appears that ESCs isolated from fertile patients do not seem to have any statistically significant alterations in the expression of the type 2 11β-HSD enzyme, regardless of treatment exposure, except for the 48h treatment with F and DHT, which resulted in a decrease in the expression of 11β-HSD2 compared to untreated cells ($p \leq 0.05$) (figure 5.20A and F). However, looking at the average results obtained, it also seems that cAMP, F and DHT co-treatment may also result in the repression of 11β-HSD2 compared to the untreated ESCs. More patients would have to be included in the study to investigate whether this effect is significant.

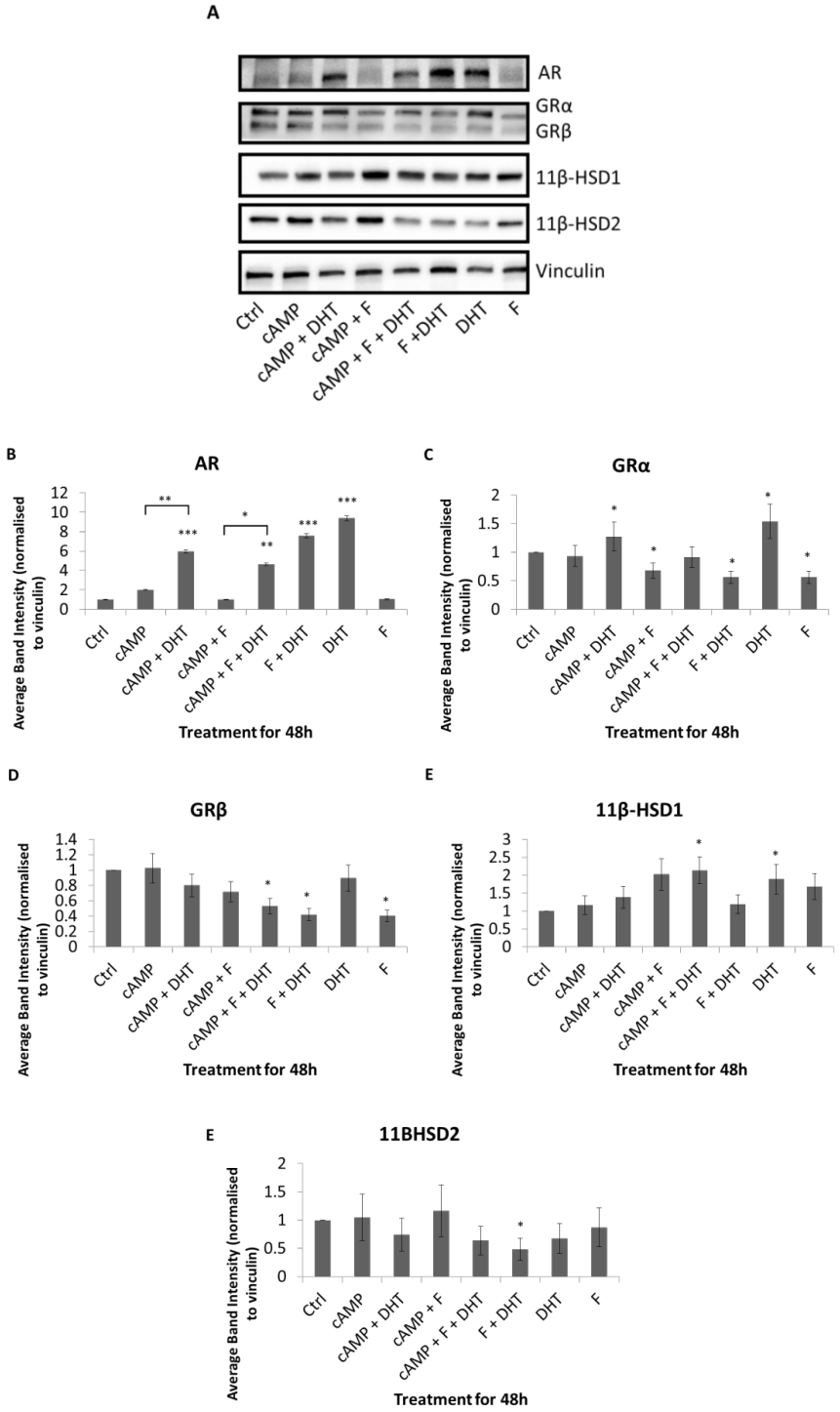


Figure 5.20 The effect of cortisol (F) and androgen exposure on the expression of AR, GR and the 11β-HSD isoforms during decidual transformation of ESCs isolated from fertile patients. A) Representative images show the western blot analysis of AR (110 kDa), GRα (95 kDa), GRβ (90 kDa), 11β-HSD1 (32 kDa), 11β-HSD2 (40 kDa) and vinculin (124 kDa) protein levels in ESCs isolated from fertile patients (n=5) after 48h treatment with either cAMP, cAMP + DHT, cAMP + F, cAMP + F + DHT, F + DHT, DHT or F alone compared to the untreated control. B-F) The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the house keeper protein; vinculin. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

These experiments were repeated with ESCs isolated from infertile patients suffering from PCOS. The immunoblot results obtained for ovPCOS patients will be described first (figure 5.21).

Immunoblot analysis revealed that treatment with cAMP + DHT, cAMP + F + DHT, F + DHT, DHT alone and F alone resulted in ESCs isolated from ovPCOS patients having a very similar level of significant induction of AR protein expression compared to untreated cells (all $p \leq 0.05$) (figure 5.21A and B). However, 48h treatment with cAMP + F resulted in a significantly enhanced expression of AR compared to both untreated cells ($p \leq 0.001$) and cells co-treated with cAMP, F + DHT ($p \leq 0.01$), whereas the opposite results were obtained for ESCs isolated from fertile patients (figure 5.20A and B). Results obtained for GRα expression also differed to those obtained for fertile patients, where it was observed that co-treatment of ESCs isolated from ovPCOS patients with cAMP, F + DHT resulted in a significant reduction of GRα protein expression compared to untreated cells ($p \leq 0.05$) (figure 5.21 A and C). Additionally, treatment of ovPCOS ESCs with F + DHT also resulted in a significant reduction of GRα protein expression ($p \leq 0.05$), where the opposite effect was observed in the fertile patients.

Interestingly, immunoblot analysis revealed that treatments do not seem to have a significant effect on GRβ isoform expression in ESC isolated from fertile patients. However, this was not the case for patients suffering from ovPCOS. It was found that co-treatment of ESCs isolated from ovPCOS patients with cAMP + DHT resulted in a significant induction of GRβ protein expression compared to cells treated with cAMP only ($p \leq 0.05$) (figure 5.21A and D). This effect was also observed on the addition of F to this treatment, where co-treatment with cAMP, F + DHT also resulted in a significant reduction in GRβ expression compared to cells treated with cAMP + F only ($p \leq 0.05$).

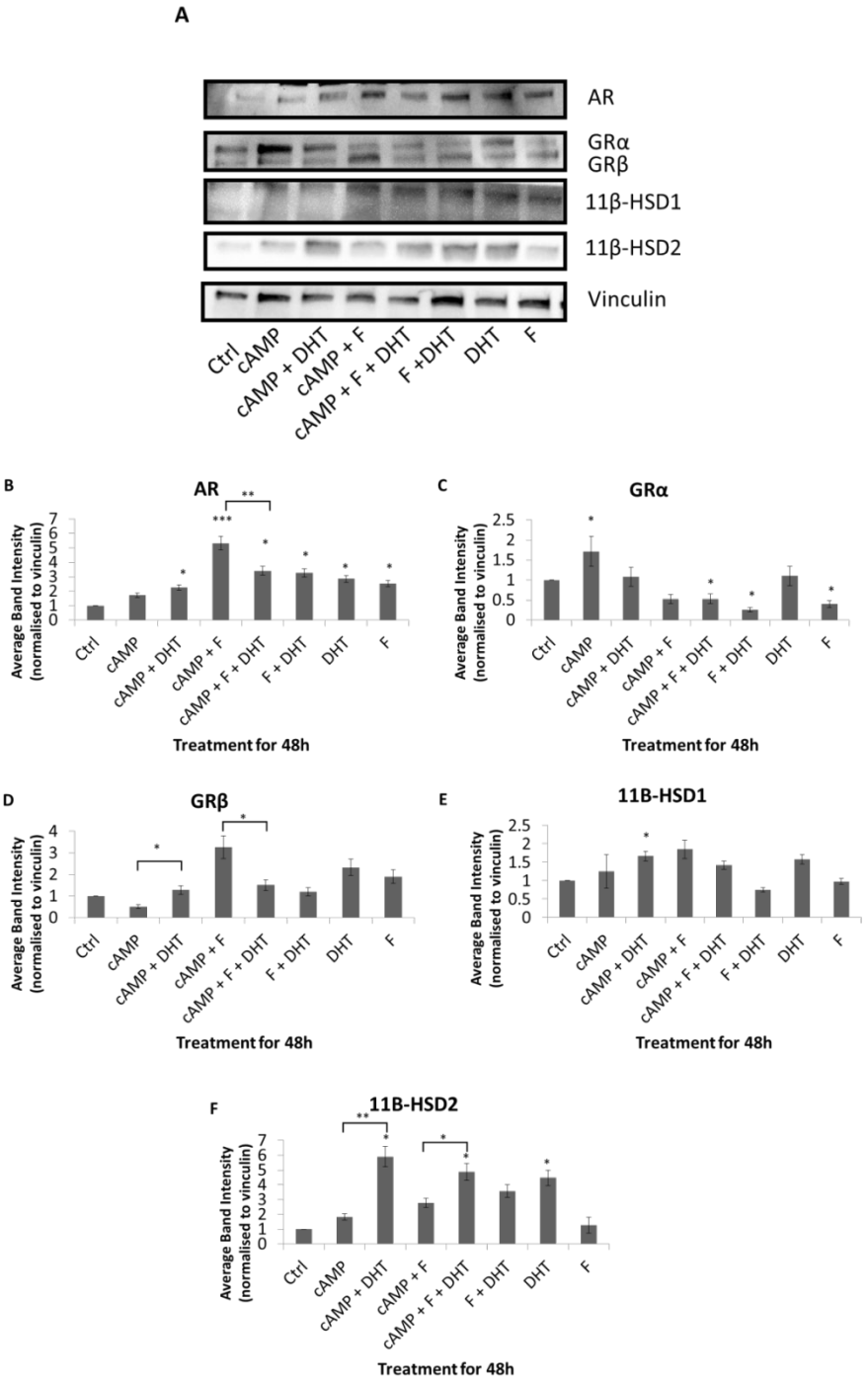


Figure 5.21 The effect of cortisol (F) and androgen exposure on the expression of AR, GR and the 11β-HSD isoforms during decidual transformation of ESCs isolated from ovPCOS patients. A) Representative images show the western blot analysis of AR (110 kDa), GRα (95kDa), GRβ (90 kDa), 11β-HSD1 (32 kDa), 11β-HSD2 (40 kDa) and vinculin (124 kDa) protein levels in ESCs isolated from ovPCOS patients (n=3) after 48h treatment with either cAMP, cAMP + DHT, cAMP + F, cAMP + F + DHT, F + DHT, DHT or F alone compared to the untreated control. B-F) The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the house keeper protein; vinculin. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

Even though opposing results were obtained for the GR isoforms when comparing ovPCOS and fertile patients, it was of interest that similar results were obtained for the 11β-HSD1 enzyme. Immunoblot analysis revealed that the only treatment group which had a significant effect on the protein expression of the type 1 isoform was cAMP + DHT ($p \leq 0.05$) (figure 5.21 A and E).

Similar to that seen in the fertile patients, treatment of ESCs isolated from ovPCOS patients with F + DHT also seemed to result in a reduction in 11β-HSD1 expression levels but this was not significant.

The pattern of expression observed for 11β-HSD2 in ESCs isolated from ovPCOS patients, remarkably followed the same pattern of expression that was seen for GRβ. Immunoblot analysis revealed that treatment of ESCs isolated from ovPCOS patients with either cAMP + DHT, cAMP + F + DHT, or DHT alone resulted in a significant increase in the expression of 11β-HSD2 expression (all $p \leq 0.05$) (figure 5.21A and F). Furthermore, co-treatment with cAMP + DHT resulted in significant increase in protein expression compared to cAMP treatment only ($p \leq 0.01$), this was mirrored on the addition of F, where co-treatment with cAMP + F + DHT also resulted in an increase in 11β-HSD2 expression compared to cAMP + F treatment only ($p \leq 0.05$) (figure 5.21 A and F).

ESCs isolated from anPCOS patients were also analysed after treatment with a combination of cortisol and androgens during decidual transformation.

Interestingly, even though ovPCOS and anPCOS patients suffer from the same syndrome, significant differences were observed in terms of protein expression levels when ESCs underwent 48h treatments. It was found that co-treatment of ESCs isolated from anPCOS patients resulted in a significant induction of AR protein expression compared to both untreated cells ($p \leq 0.01$) and to cells treated with the decidualization stimulus only ($p \leq 0.05$) (figure 5.22A and B), which was not observed in patients suffering from ovPCOS. Interestingly, unlike ovPCOS patients, treatment of ESCs isolated from anPCOS patients with cAMP + F did not result in an increase in AR expression compared to untreated cells. However, on the addition of DHT (i.e. those treated with cAMP, F + DHT), there was a significant induction in AR protein expression compared to both untreated control cells ($p \leq 0.01$) and also those treated with cAMP + F only ($p \leq 0.05$). Similar results were obtained to ovPCOS patients, when the ESCs isolated from anPCOS patients were treated with either F + DHT or DHT alone, where a significant induction in AR expression was exhibited ($p \leq 0.01$) (figure 5.22 A and B), but this induction was to a much greater extent. For example, treatment of ESCs isolated from anPCOS patients with F + DHT resulted in an

approximately six fold increase in protein expression in terms of immunoblot band intensity compared to an approximately three fold increase of AR expression in ovPCOS patients.

Alternatively, a similar pattern of GRα pattern in anPCOS patients was observed compared to ovPCOS patients. It was found that similarly to ESCs isolated from ovPCOS patients, treatment of ESCs isolated from anPCOS patients with either cAMP + F + DHT or F + DHT resulted in a significant reduction of GRα protein expression ($p \leq 0.01$ and $p \leq 0.05$ respectively) (figure 5.22 A and C). Unlike the results obtained for AR, there were no significant alterations in GRα protein expression in cells treated with cAMP + DHT compared to the untreated cells or cells treated with cAMP only and furthermore, treatment with cAMP + F + DHT did also not have a significant effect on AR expression compared to cells treated with cAMP + F only.

Interestingly, an alternative pattern of expression was observed for GRβ. It was found that unlike ovPCOS patients, treatment of the ESCs with cAMP + DHT actually resulted in a significant decrease in GRβ expression compared to both untreated cells ($p \leq 0.01$) and cells treated with the decidualization stimulus only ($p \leq 0.05$) (figure 5.22 A and D), whereas the opposite effect on GRβ protein expression was observed in ovPCOS patients. However, when ESCs isolated from anPCOS patients were treated with cAMP + F + DHT, a similar effect was observed to that seen in ovPCOS patients. For example, cells co-treated with cAMP + F + DHT resulted in a reduction in GRβ expression compared to control cells ($p \leq 0.01$) which on average was lower than values obtained for cells treated with cAMP + F only, however this was not statistically significant unlike the results obtained for ovPCOS patients, suggesting that this effect is not as powerful. Interestingly, treatment of ESCs isolated from anPCOS patients with either F + DHT or DHT alone also resulted in a significant reduction in GRβ expression compared to untreated cells ($p \leq 0.05$), suggesting a possible interplay between the GR and AR signalling pathways (figure 5.22 A and D).

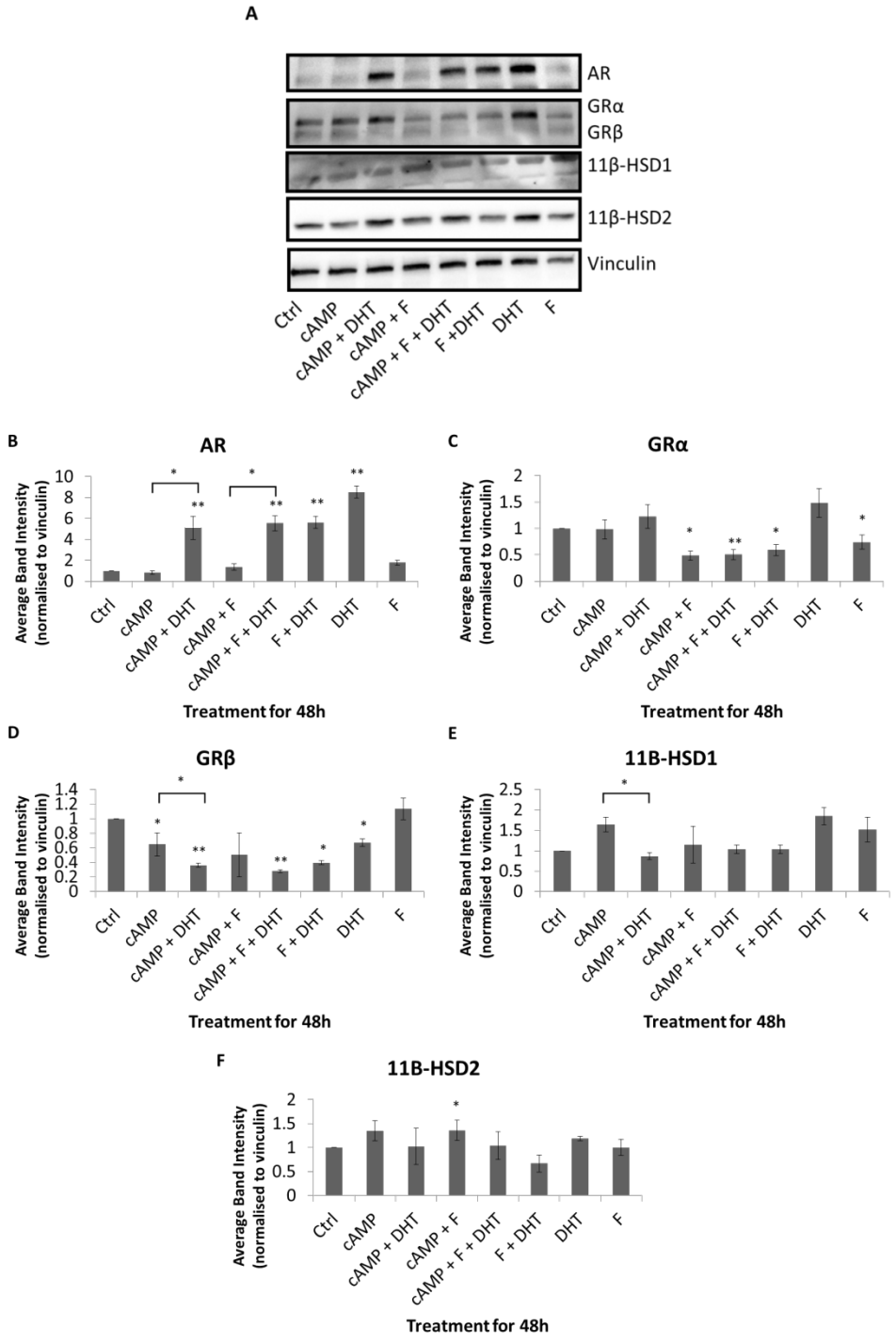


Figure 5.22 The effect of cortisol (F) and androgen exposure on the expression of AR, GR and the 11β-HSD isoforms during decidual transformation of ESCs isolated from anPCOS patients. A) Representative images show the western blot analysis of AR (110 kDa), GRα (95 kDa), GRβ (90 kDa), 11β-HSD1 (32 kDa), 11β-HSD2 (40 kDa) and vinculin (124 kDa) protein levels in ESCs isolated from anPCOS patients (n=5) after 48h treatment with either cAMP, cAMP + DHT, cAMP + F, cAMP + F + DHT, F + DHT, DHT or F alone compared to the untreated control. B-F) The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the house keeper protein; vinculin. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

To gain some additional information about the effect of androgens on the GR signalling pathway during decidual transformation, it was also decided to investigate the expression of the 11βHSD isoforms as was conducted for the fertile and ovPCOS patients. It was found that the expression of 11β-HSD1 followed a similar pattern to those seen for GRβ, where treatment with cAMP + DHT resulted in a significant decrease in the protein expression levels of the type 1 isoform compared to cells treated with cAMP only ($p \leq 0.05$) (figure 5.22A and E). Also, treatment with cAMP + F + DHT also resulted in on average an overall decrease in 11β-HSD1 protein expression compared to cells treated with cAMP + F only, but this was not significant. Unlike GRβ, treatment of ESCs isolated from anPCOS patients with F + DHT or DHT alone, resulted in a stable expression of 11β-HSD1 where no significant impact on protein expression was observed (figure 5.22A and E).

Surprisingly, extremely different results were obtained for 11β-HSD2 expression in ESCs isolated from anPCOS patients in response to cortisol, androgens and the decidualization stimulus compared to results obtained for ovPCOS patients. It was found that unlike ovPCOS, 11β-HSD2 protein expression does not seem to be induced on the addition of DHT during decidual transformation, either in the absence or presence of the stress hormone cortisol (figure 5.22A and F). The only significant result obtained was in response to treatment with cAMP + F, which has been described previously in this chapter.

5.2.4.3 The effect of cortisol and androgens on the expression of potential GR targets during decidual transformation

Due to the aberrant expression of the key proteins involved in the GR signalling pathway in PCOS patients in the presence of cortisol and androgens during decidual transformation, it was decided to investigate whether cortisol and androgen exposure was having a significant impact on the expression of potential GR signalling pathway target proteins during decidual transformation.

Immunoblot analysis was initially carried out on ESCs isolated from fertile patients, which had undergone 48h treatment regimens and compared to both untreated control cells and to ESCs isolated from anPCOS patients. Immunoblot analysis revealed both WT1 and FKBP5 protein expression was induced when ESCs isolated from fertile patients underwent *in vitro* decidualization ($p \leq 0.05$) (figure 5.23A, B and C). Additionally, treatment of the cells with cAMP + F also resulted in an induction of both WT1 and FKBP5 at the protein level (not significant), but these expression levels were lower than that seen in cells treated with cAMP only. Interestingly, it was found that co-treatment with cAMP + DHT resulted in a

significant decrease in the levels of FKBP5 protein expression compared to ESCs treated with cAMP only ($p \leq 0.05$) (figure 5.23A and C). Additionally, co-treatment with cAMP, F + DHT also resulted in a significant reduction in expression of FKBP5 compared to cells treated with cAMP + F only ($p \leq 0.01$).

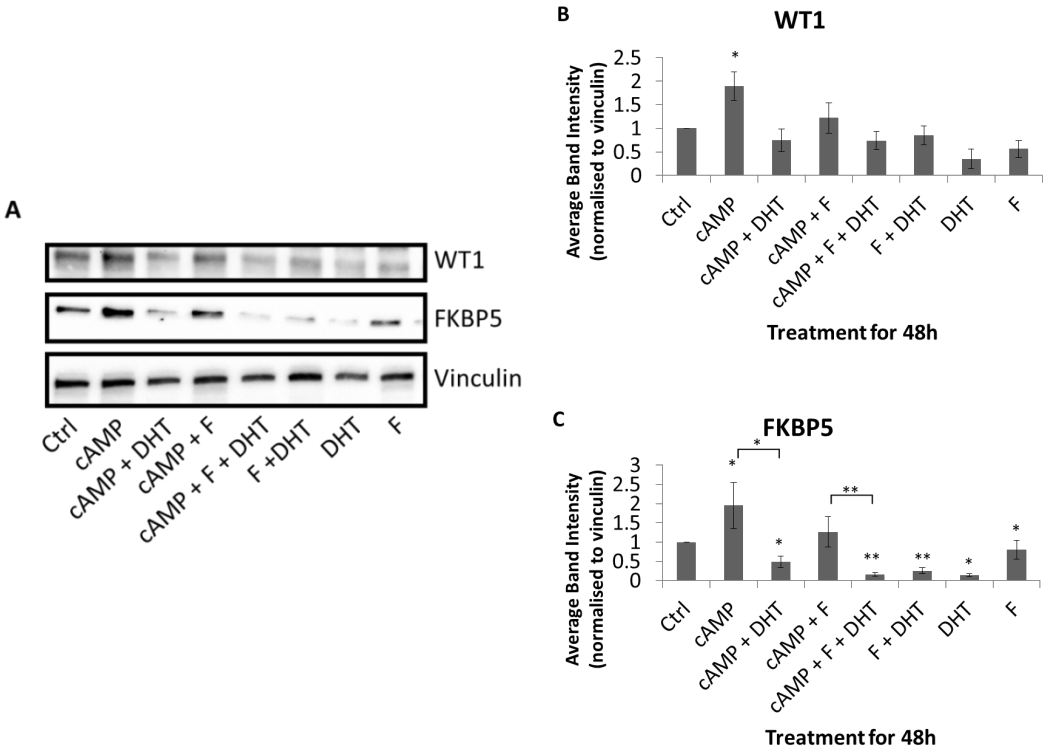


Figure 5.23 The effect of cortisol (F) and androgen exposure on the expression of WT1 and FKBP5 during decidual transformation of ESCs isolated from fertile patients. A) Representative images show the western blot analysis of WT1 (52 kDa), FKBP5 (51 kDa) and vinculin (124 kDa) protein levels in ESCs isolated from fertile patients (n=5) after 48h treatment with either cAMP, cAMP + DHT, cAMP + F, cAMP + F + DHT, F + DHT, DHT or F alone compared to the untreated control. B-F) The intensity of the immunoblot bands was measured quantitatively using Image Lab software and normalised to the housekeeper protein; vinculin. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ are considered significant.

Furthermore, treatment of the ESCs isolated from fertile patients with either F + DHT or DHT alone also resulted in a significantly lower expression of FKBP5 compared to control cells ($p \leq 0.01$ and $p \leq 0.05$) which was comparable to the expression levels seen in the cAMP, F + DHT treatment groups. This suggests that the presence of androgens has an inhibitory effect on FKBP5 and WT1 in ESCs isolated from fertile patients in the presence of absence of the stress hormone cortisol.

Immunoblot analysis was further carried on protein extracted from ESCs isolated from patients suffering from ovPCOS.

It was found that 48h treatment with cAMP + DHT, significantly decreased the WT1 protein expression levels compared to both the untreated control cells and cells treated with cAMP only ($p \leq 0.05$) (figure 5.24A and B). Furthermore, treatment of ESCs isolated from ovPCOS patients with cAMP + F + DHT also resulted in a significant reduction in endometrial expression levels compared to cells treated with cAMP + F only and untreated cells ($p \leq 0.05$ and $p \leq 0.01$ respectively). Unlike the results obtained for fertile patients, treatment of ESCs isolated from ovPCOS patients with cAMP or cAMP + F, did not result in a significant induction in the expression of FKBP5 compared to control cells. Treatment with cAMP only, actually appears to result in a slight reduction of FKBP5 expression compared to control cells. The addition of DHT during decidualization in both the presence and absence of F resulted in no significant alterations in FKBP5 protein expression.

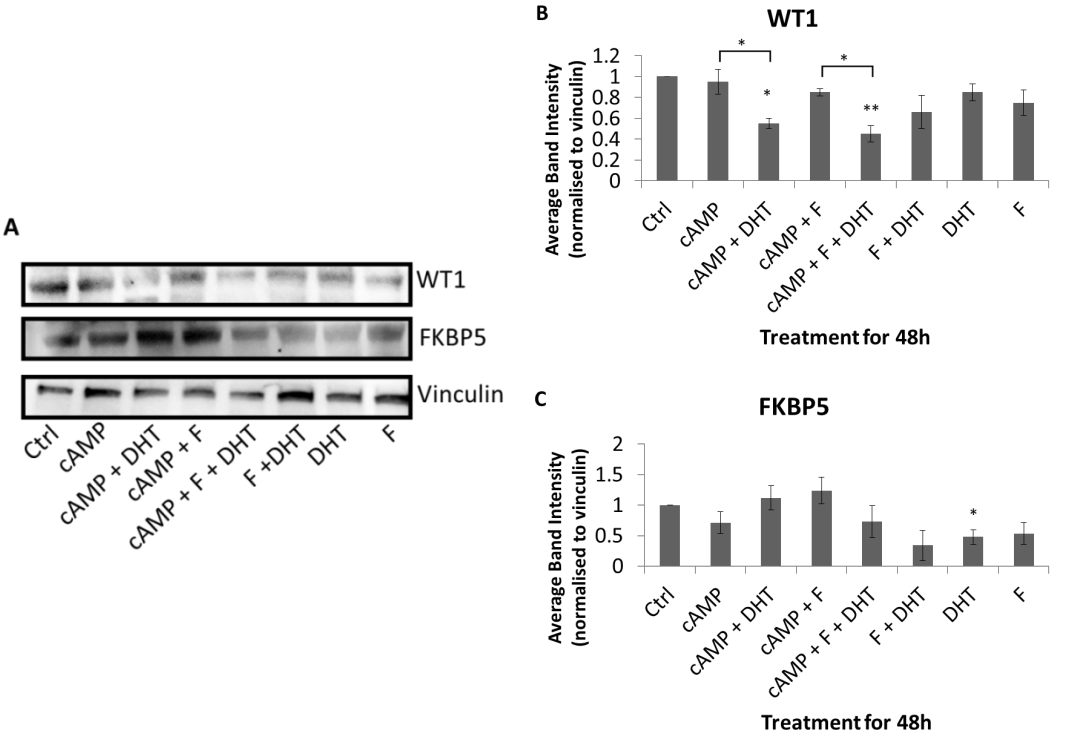


Figure 5.24 The effect of cortisol (F) and androgen exposure on the expression of WT1 and FKBP5 during decidual transformation of ESCs isolated from ovPCOS patients. A) Representative images show the western blot analysis of WT1 (52 kDa), FKBP5 (51 kDa) and vinculin (124 kDa) protein levels in ESCs isolated from ovPCOS patients (n=3) after 48h treatment with either cAMP, cAMP + DHT, cAMP + F, cAMP + F + DHT, F + DHT, DHT or F alone compared to the untreated control. B-F) The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the house keeper protein; vinculin. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ are considered significant.

Treatment of the ESCs with F + DHT or F resulted in a lower expression of FKBP5 protein compared to the control cells but this was not deemed to be statistically significant. However, treatment with DHT only, resulted in a significant reduction of FKBP5 expression compared to untreated control cells ($p \leq 0.05$) (figure 5.24A and C).

Lastly, the immunoblot analysis was also carried out on ESCs isolated from anPCOS patients, where the results obtained differ from both fertile and ovPCOS patients. Immunoblot analysis revealed that cAMP treatment resulted in a significant decrease in WT1 protein expression levels as previously mentioned compared to untreated cells ($p \leq 0.05$) (figure 5.25A and B). On addition of the DHT and cAMP combination there was a further decrease in WT1 protein expression levels observed compared to the control cells and cells treated with cAMP only ($p \leq 0.01$ and $p \leq 0.05$ respectively). As previously described, co-treatment of the ESCs isolated from anPCOS patients with cAMP + F resulted in a significantly reduced expression of WT1 compared to control cells, but this was further decreased with the addition of DHT ($p \leq 0.001$) (figure 5.25A and B). Treatment with either F + DHT, DHT alone or F alone also resulted in an overall decrease in the WT1 expression compared to control cells ($p \leq 0.05$), but this inhibition of expression was not to the same extent seen in cells co-treated with cAMP + F + DHT.

On the other hand, treatment of ESCs isolated from anPCOS patients with cAMP, had no effect on the expression of FKBP5 (figure 5.25A and C). The addition of DHT during *in vitro* decidualization also did not result in any alterations of FKBP5 levels. In contrast, treatment of the ESCs with cAMP + F resulted in a significant increase in the expression of FKBP5 ($p \leq 0.05$). However, the addition of DHT in combination with cAMP + F, it was observed that FKBP5 protein expression was repressed compared to control cells ($p \leq 0.05$) and cells treated with cAMP + F only ($p \leq 0.05$). Treatment of ESCs isolated from anPCOS patients with F + DHT and F alone also resulted in a significant decrease in FKBP5 protein expression ($p \leq 0.01$ and $p \leq 0.05$ respectively).

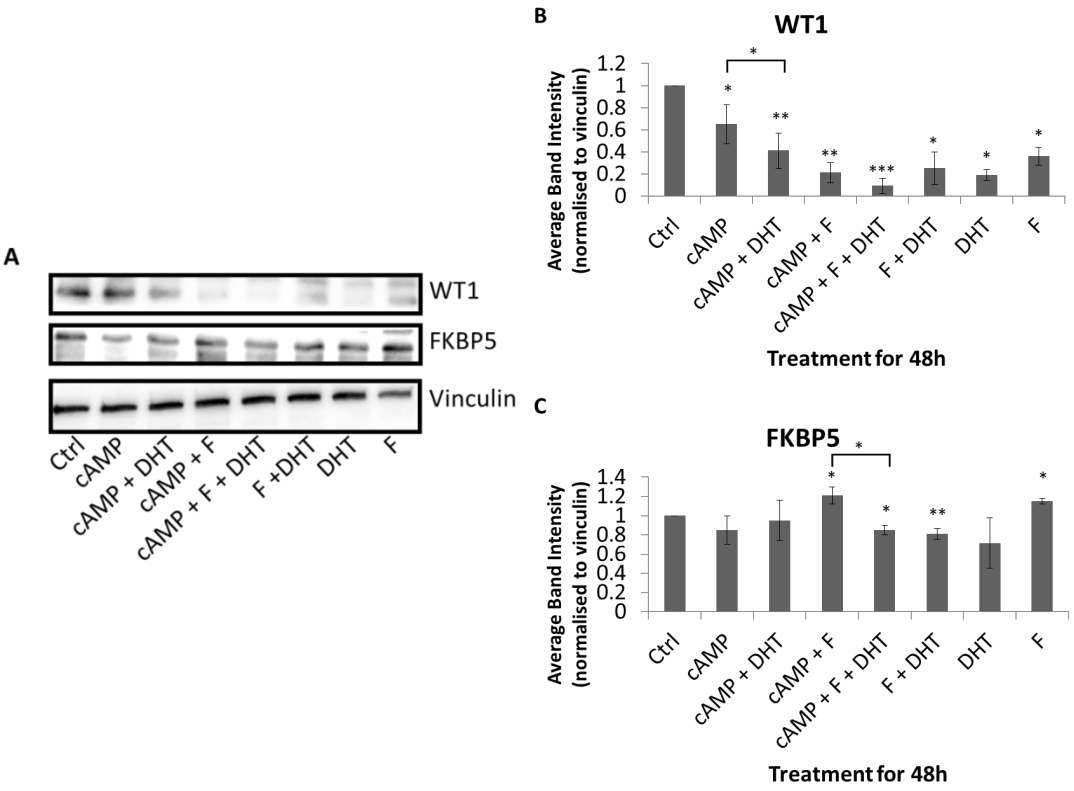


Figure 5.25 The effect of cortisol (F) and androgen exposure on the expression of WT1 and FKBP5 during decidual transformation of ESCs isolated from anPCOS patients. A) Representative images show the western blot analysis of WT1 (52 kDa), FKBP5 (51 kDa) and vinculin (124 kDa) protein levels in ESCs isolated from anPCOS patients (n=5) after 48h treatment with either cAMP, cAMP + DHT, cAMP + F, cAMP + F + DHT, F + DHT, DHT or F alone compared to the untreated control. B-F) The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the house keeper protein; vinculin. Statistical analysis was performed using the ANOVA statistical test followed by the Student's t test. Values are averaged and bars indicate ± SD. *p≤0.05, **p≤0.01 are considered significant.

5.2.5 The effect of progesterone and the progesterone receptor (PR) on the GR signalling pathway in infertile patients

In addition to androgens and the AR, it was also decided to investigate the interaction of progesterone and PR on the GR signalling pathway in the ESCs isolated from infertile patients particularly during *in vitro* decidualization.

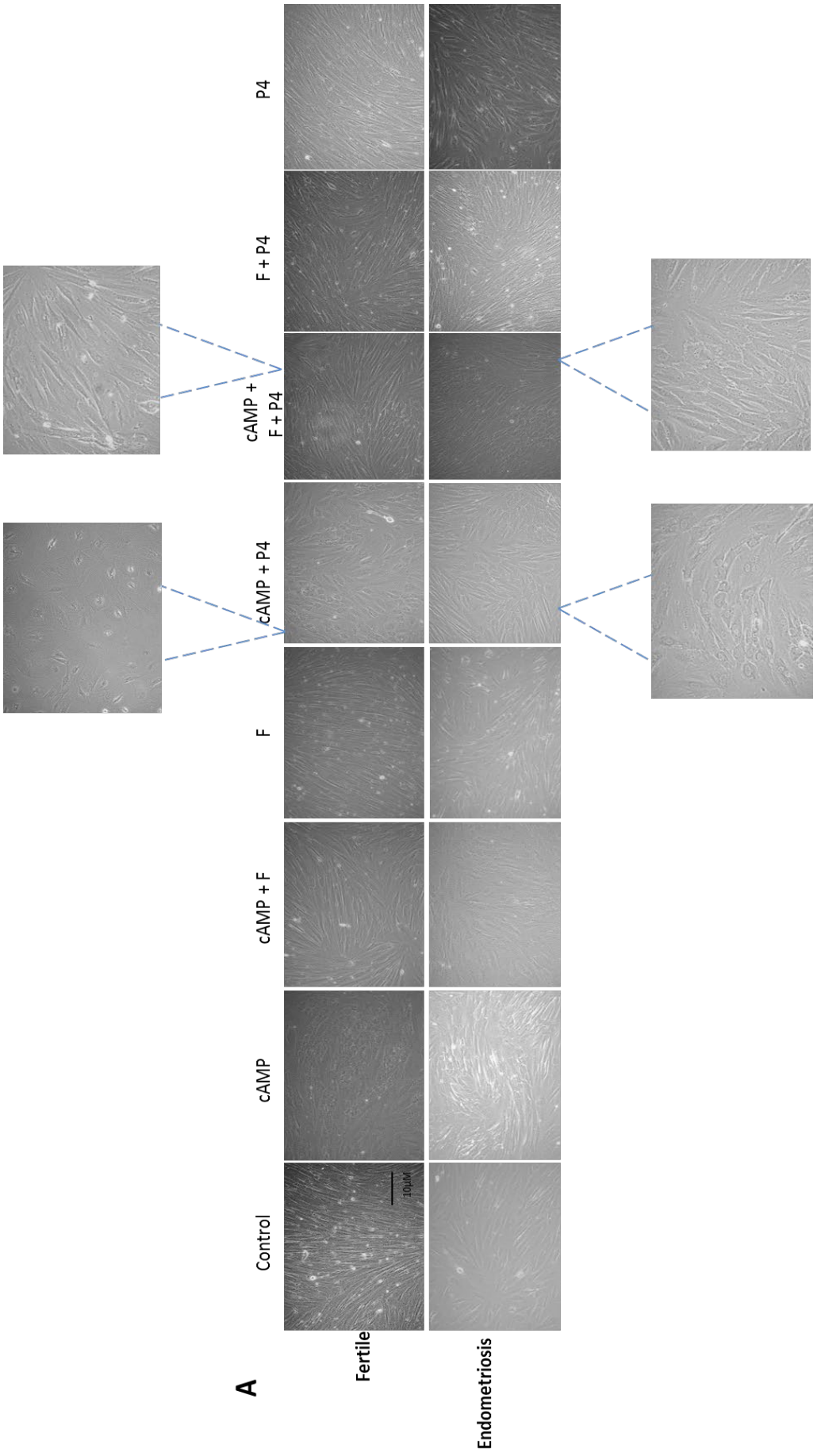
All experiments were carried out in both ESCs isolated from patients suffering from endometriosis and compared to ESCs isolated from fertile patients. Endometriosis patients were chosen for these *in vitro* experiments as it is known that eutopic endometrial cells isolated from a subset of endometriosis patients suffer from progesterone resistance and altered progesterone signalling pathways (Patel et al, 2017). This results in endometriosis

patients exhibiting defects in other sex steroid hormone pathways, including the estrogen signalling pathway (Bulun et al, 2006).

Experiments presented in section 5.2.4 were repeated, but all DHT treatments were substituted by progesterone (P_4) treatment.

5.2.5.1 The effect of cortisol and progesterone on cell morphology and the expression of key decidual markers during decidual transformation

As described previously, ESCs isolated from both fertile and endometriosis patients were cultured and treated with either cAMP, cAMP F, F alone, cAMP + P_4, cAMP + F + P_4, F + P_4 or P_4 alone and compared to cells which had not undergone any treatment. Images of the cell monolayers were obtained using inverted light microscopy and these images were used to quantitatively measure the average circularity values of these cells. As described previously in this chapter, it was found that cAMP treatment, resulted in a significant increase in average circularity values of ESCs isolated from both fertile and endometriosis patients ($p \leq 0.001$ and $p \leq 0.01$), (figure 5.26A and B). Whereas treatment of ESCs isolated from fertile and endometriosis patients with cAMP + F resulted in a significant reduction in cell circularity values compared to cells treated with cAMP only ($p \leq 0.001$ and $p \leq 0.05$ respectively). As expected, treatment of ESCs isolated from both fertile and endometriosis patients with cAMP + P_4 resulted in a significant increase in cell circularity values compared to control cells ($p \leq 0.01$ and $p \leq 0.05$) (figure 5.26B), which can also be seen in the light microscopy images obtained (figure 5.26A). However, it is important to note that cAMP + P4 + F treatment of endometriosis ESCs results in a lower circularity value of the cells measured compared to fertile cells. Similar to the results obtained in the absence of P4, it was discovered that treatment of ESCs isolated from fertile patients with cAMP + P4 + F, results in a significant decrease in the average cell circularity value compared to cells treated with cAMP + P4 only ($p \leq 0.01$), whereas this effect was not reciprocated in ESCs isolated from endometriosis patients (figure 5.26 A and B).


A
Fertile
Endometriosis
Control
cAMP
cAMP + F
F
cAMP + P4
cAMP + F + P4
F + P4
P4
10µM

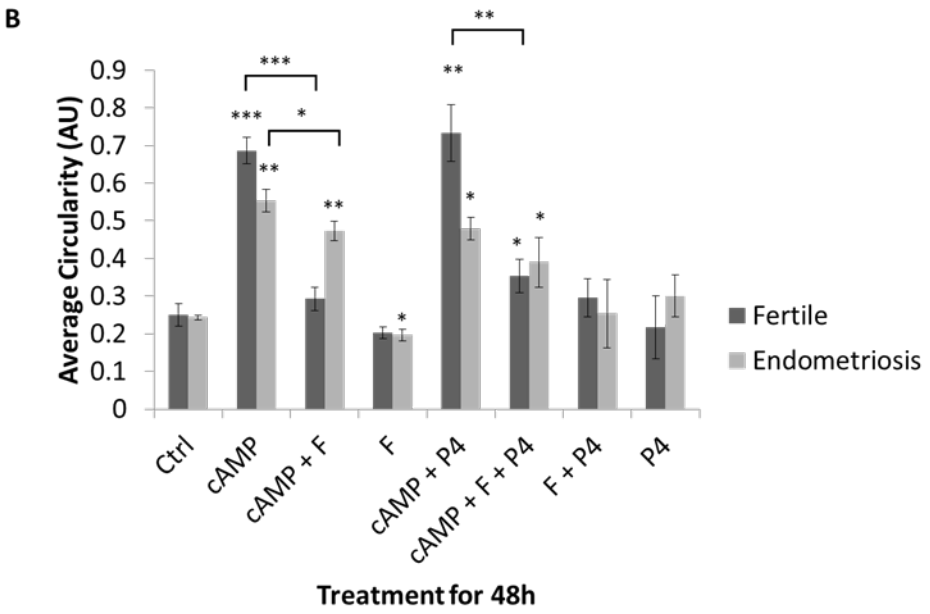


Figure 5.26A and 5.26B. The effect of cortisol (F) and progesterone (P4) exposure on the morphology and circularity values of ESCs isolated from infertile patients suffering from endometriosis compared to fertile patients during decidual transformation. A) Representative images taken of the cell monolayers after 48 h of treatments using inverted light microscopy (Zeiss) at an x10 objective. Scale bar indicates 10μM. These images were then used for Image J analysis to gain a quantitative measure of cell morphology. Images taken at a x20 objective were also included as insets indicated by dashed lines. B) Thirty cells were chosen at random from each image taken of ESCs isolated from fertile patients (n=5) and endometriosis patients (n=6). Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

ELISA analysis of the key decidual markers was further carried out on the conditioned media isolated from the cells, to determine whether the morphological analysis presented in figure 5.26 was indicative of an effect on *in vitro* decidualization (figure 5.27).

It was found that ELISA analysis supported the morphological data obtained, where treatment of the ESCs isolated from fertile and endometriosis patients resulted in comparable levels of secreted PRL and IGFBP-1 being present compared to cells treated with cAMP only. Furthermore, it was found that treatment of ESCs isolated from fertile patients only with cAMP + F + P4 resulted in a significant decrease in the secreted levels of PRL compared to cells co-treated with cAMP + P4 only (p≤0.01) (figure 5.27A). However, this effect was not seen for the secreted levels of IGFBP1 (figure 5.27 B). Treatment of ESCs isolated from endometriosis with cAMP + F + P4 had no effect on the secreted PRL levels compared to cAMP + P4 treatment only, whereas a significant decrease in the expression of IGFBP-1 protein was observed (p≤0.05) (figure 5.27B). It is also important to note that the addition of P4 to ESCs isolated from fertile patients which are co-treated with cAMP + F resulted in a significant increase in IGFBP-1 expression only compared to cells treated with cAMP + F only (p≤0.01), whereas the opposite effect was obtained for endometriosis patients. Where cAMP + F + P4 treatment resulted in a significant reduction

of IGFBP-1 expression at the protein level compared endometriosis cells treated with cAMP + F only ($p \leq 0.05$) (figure 5.27B). This ELISA analysis fully supports the morphological data obtained.

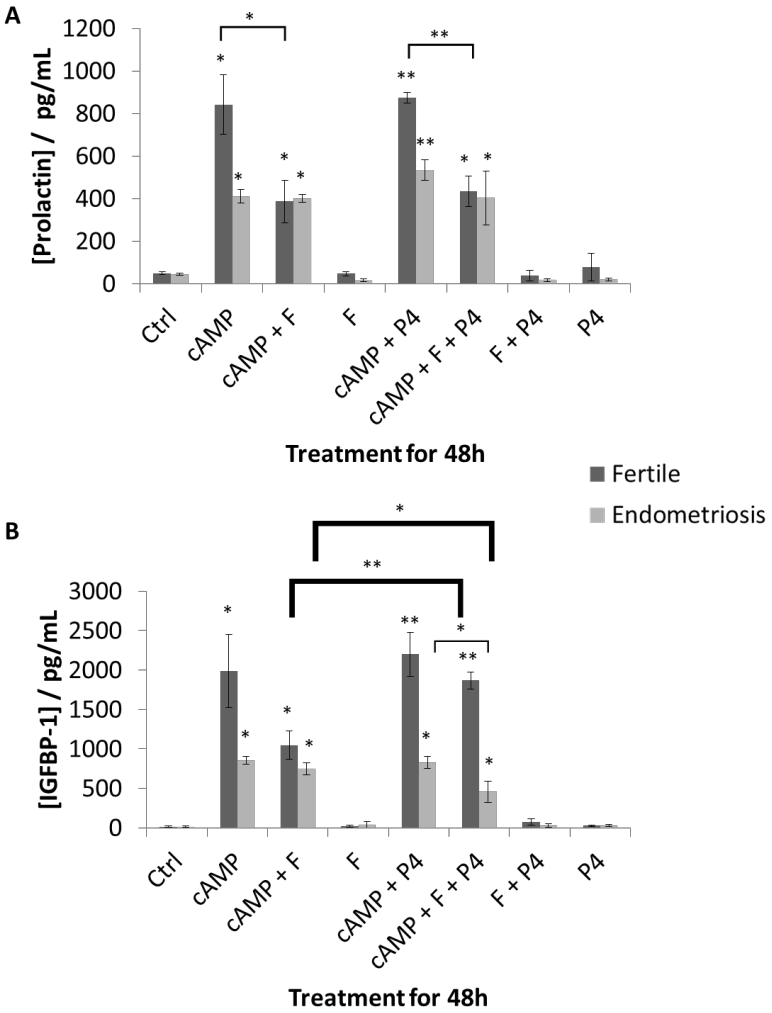


Figure 5.27. The effect of cortisol (F) and progesterone (P4) exposure on the levels of secreted decidual markers – prolactin (A) and IGFBP-1 (B) from ESCs isolated from infertile patients suffering from endometriosis and compared to fertile patients during decidual transformation. Media was extracted after ESCs isolated from fertile patients (n=5) and endometriosis patients (n=6) after 48 h of treatment with either cAMP, cAMP and F, F alone, cAMP and P4, cAMP and F and P4, F and P4 orP4 alone and compared to their corresponding untreated cells. ELISA experiments were carried out on the conditioned media to establish the concentration of key proteins at the secreted level. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ are considered significant.

5.2.5.2 The effect of cortisol and progesterone on the expression of GR, PR and 11β-HSD during decidual transformation

To further investigate the interaction between GR and PR signalling pathways, it was decided to investigate the effect of cortisol and progesterone exposure on the expression of key proteins involved in the GR signalling pathway and also PR. Experiments were first conducted on ESCs isolated from fertile patients and protein isolated from endometriosis patients was included on the immunoblots to enable a direct comparison. Immunoblot analysis revealed interesting results, where it was shown that treatment of fertile ESCs with cAMP + F resulted in a significant induction of PR protein expression compared to untreated cells ($p \leq 0.001$) (figure 5.28A and B). However, on addition of P4 in combination with cAMP + F, it was found that the expression of PR was significantly down-regulated compared to cells treated with cAMP + F only ($p \leq 0.05$), but remained at a higher level of expression compared to control cells ($p \leq 0.05$). No other treatment resulted in any significant alterations of PR protein expression.

The effects demonstrated on PR expression, were not reciprocated for GR protein expression in ESCs isolated from fertile patients. It was demonstrated that cAMP treatment, overall results in an increase in the expression of GRα at the protein level, which was not statistically significant (figure 5.28A and C). It was interesting to observe that co-treatment with both cAMP + P4 and cAMP + F both resulted in a significant decrease in GRα protein expression compared to cells treated with cAMP only ($p \leq 0.05$). This reduced expression of GRα was maintained when fertile ESCs were treated with cAMP, F + P4 or F + P4 alone (figure 5.28 A and C). This effect was not observed for GRβ expression, as only the cAMP, F + P4 treatment group had a significantly reduced expression of GRβ compared to both cells treated with cAMP + F only and untreated control cells ($p \leq 0.05$) (figure 5.28A and D). Interestingly, in the absence of cAMP – F + P4 co-treatment still resulted in a significant decrease in GRβ protein expression.

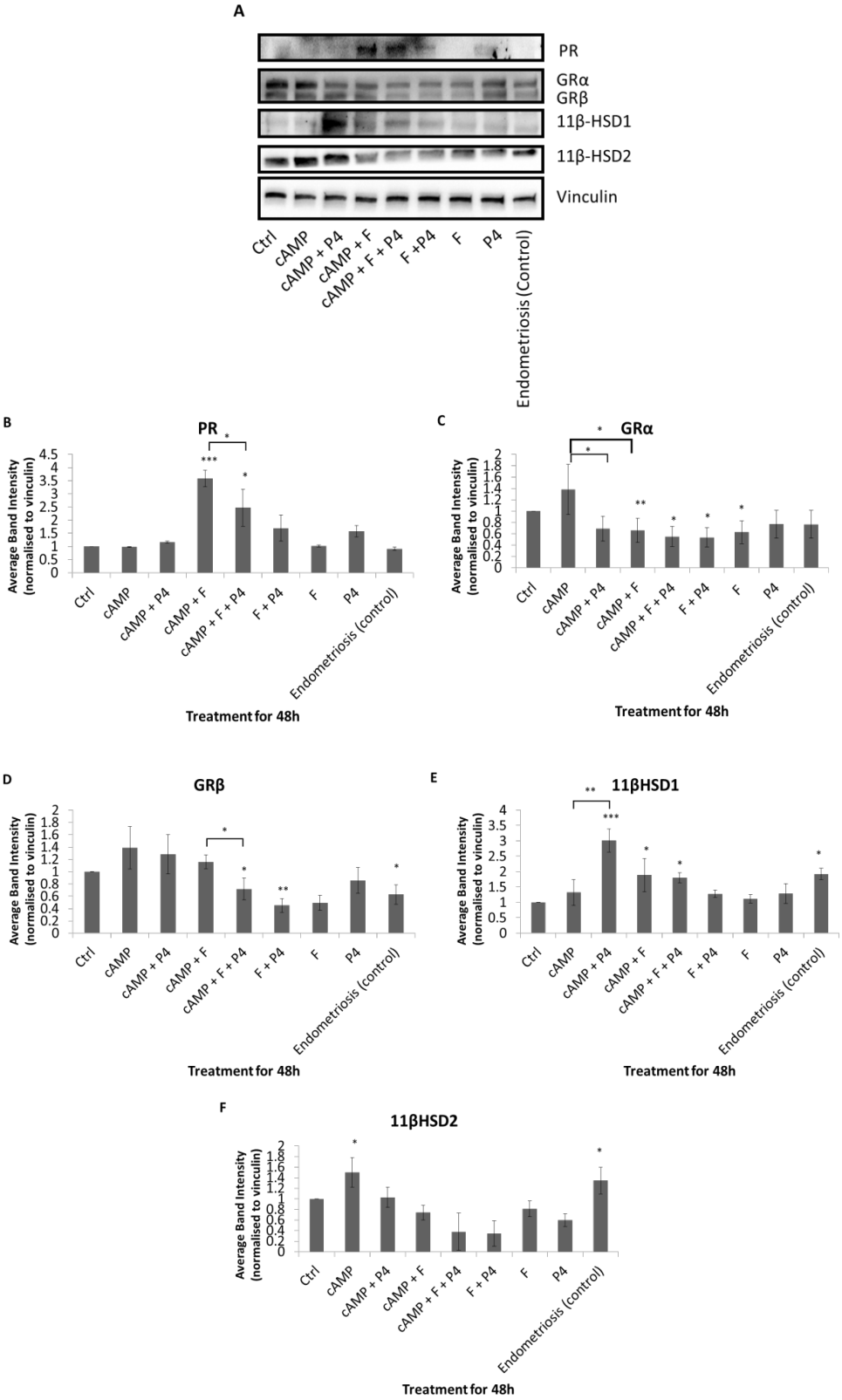


Figure 5.28 The effect of cortisol (F) and progesterone (P4) exposure on the expression of PR, GR and the 11β-HSD isoforms during decidual transformation of ESCs isolated from fertile patients. A) Representative images show the western blot analysis of PR (116 kDa), GRα (95 kDa), GRβ (90 kDa), 11β-HSD1 (32 kDa), 11β-HSD2 (40 kDa) and vinculin (124 kDa) protein levels in ESCs isolated from fertile patients (n=5) after 48h treatment with either cAMP, cAMP + P4, cAMP + F, cAMP + F + P4, F + P4, P4 or F alone compared to the untreated control. B-F) The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the house keeper protein; vinculin. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

Immunoblot analysis was also carried out on the 11βHSD enzyme isoforms, due to their critical role in the GR signalling pathway. It was observed that treatment of ESCs isolated from fertile patients with cAMP + P4 resulted in a significant increase in the expression of the type 1 isoform only compared to cAMP treatment only and cells which remained untreated ($p \leq 0.01$ and $p \leq 0.001$) (figure 5.28A and E). Interestingly, co-treatment with cAMP + F also resulted in a significant increase in 11β-HSD1 protein expression, however the addition of P4 in combination with these treatments did not have a significant effect on the type 1 isoform expression compared to cells treated with cAMP + F only. In terms of 11β-HSD2 protein expression, only cAMP treatment resulted in a significant alteration in protein expression compared to untreated control cells ($p \leq 0.05$) (figure 5.28A and F).

These experiments were further repeated with ESCs isolated from endometriosis patients in addition to the inclusion of protein isolated from fertile patients being run simultaneously on the immunoblots to allow for a direct comparison. Contrary to the results obtained for fertile patients, it was found that cAMP + F treatment did not result in a significant induction of PR expression. However, co-treatment with cAMP, F + P4 resulted in a great increase in the protein expression levels of PR compared to both untreated control cells and cells treated with cAMP + F only ($p \leq 0.001$ and $p \leq 0.01$) (figure 5.29A and B). Additionally, it was also found that 48h treatment of endometriosis ESCs with F + P4 also resulted in a significant induction of PR protein expression levels compared to untreated cells ($p \leq 0.01$).

As seen in the fertile patients, it was shown that GR had a distinct expression pattern in response to hormonal treatments compared to PR expression in endometriosis patients. Similar to the levels observed in the fertile patients, it was observed that cAMP treatment resulted in a significant increase in GRα protein expression only compared to control cells ($p \leq 0.01$) (figure 5.29A and C). However, co-treatment with cAMP + P4 did not result in any decrease in GRα protein expression; this treatment regime actually resulted in comparable protein levels of GRα compared to cAMP treatment only. Treatment with cAMP, F + P4 did also not result in any decreases in GRα protein expression compared to untreated control cells. Whereas, on the removal of cAMP – treatment with F + P4 resulted in a significant decrease in GRα expression levels comparable to that seen in the fertile patients ($p \leq 0.05$). Alternative results were also obtained for GRβ protein expression, where it was found that only cAMP + F co-treatment resulted in a significant reduction in the expression of GRβ ($p \leq 0.05$) compared to cAMP treatment only (figure 5.29A and D).

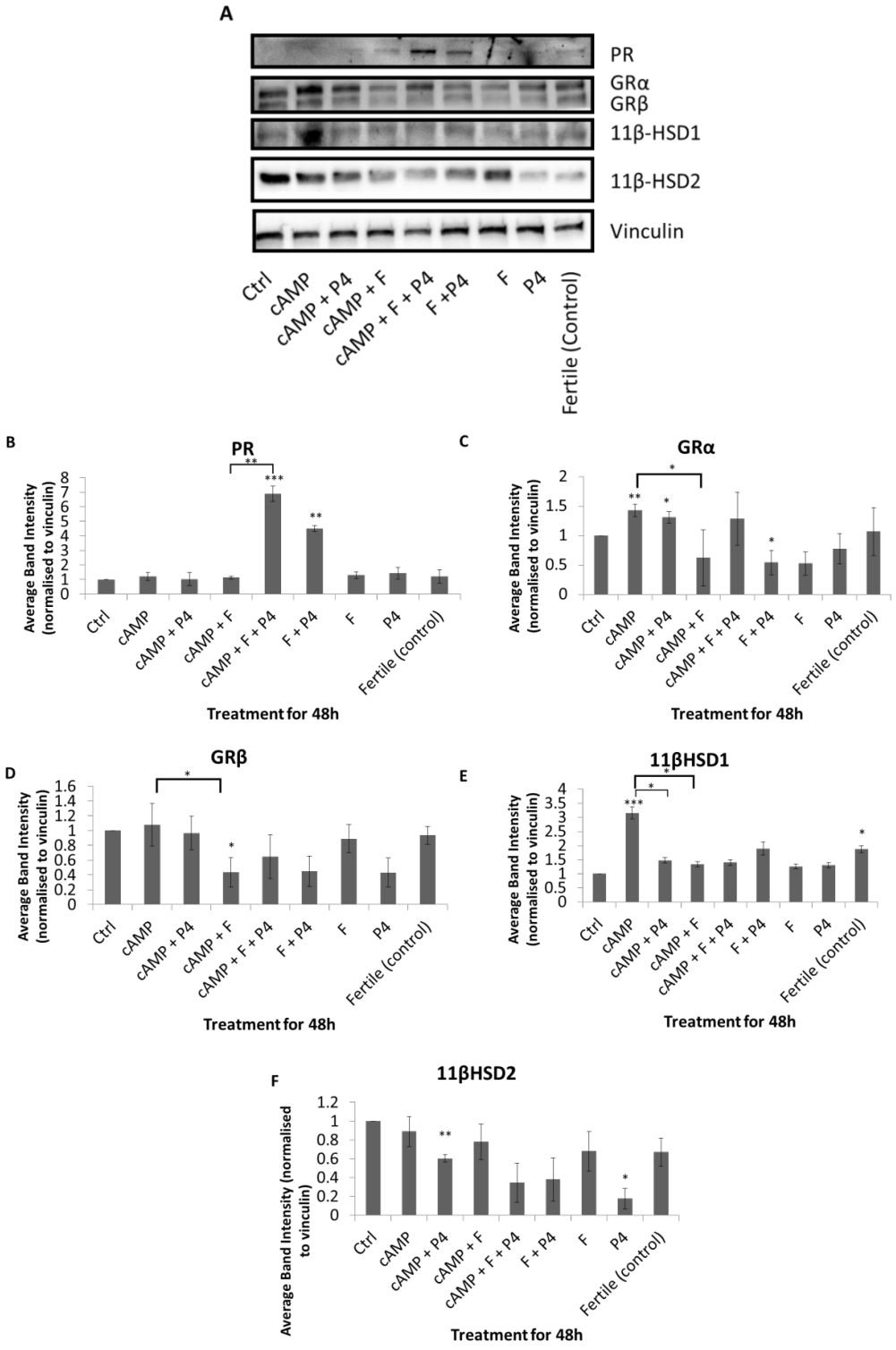


Figure 5.29 The effect of cortisol (F) and progesterone (P4) exposure on the expression of PR, GR and the 11β-HSD isoforms during decidual transformation of ESCs isolated from endometriosis patients. A) Representative images show the western blot analysis of PR (116 kDa), GRα (95 kDa), GRβ (90 kDa), 11β-HSD1 (32 kDa), 11β-HSD2 (40 kDa) and vinculin (124 kDa) protein levels in ESCs isolated from endometriosis patients (n=6) after 48h treatment with either cAMP, cAMP + P4, cAMP + F, cAMP + F + P4, F + P4, P4 or F alone compared to the untreated control. B-F) The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the house keeper protein; vinculin. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

In addition to the hormone receptors; GR and PR, immunoblot analysis was also carried out to determine the protein expression levels of the 11β-HSD enzymes in endometriosis patients. It was found that *in vitro* decidualization resulted in a significant increase in 11β-HSD1 protein expression compared to untreated cells ($p \leq 0.001$), cells treated with cAMP + P4 only ($p \leq 0.05$) and cells treated with cAMP + F only ($p \leq 0.05$) (figure 5.29A and E). However, in terms of 11β-HSD2 protein expression, only 48h treatment with cAMP + P4 and P4 only resulted in a significant decrease in 11β-HSD2 protein expression compared to untreated control cells ($p \leq 0.01$ and $p \leq 0.05$) (figure 5.29A and F). These results demonstrate that the 11β-HSD isoforms, also exhibit a distinct expression pattern in ESCs isolated from endometriosis patients compared to fertile patients in response to hormonal treatment during decidualization.

5.2.5.3 The effect of cortisol and progesterone on the expression of potential GR targets during decidual transformation

The effect of cortisol and progesterone on the GR signalling pathway and decidual transformation was further investigated in ESCs isolated from fertile and endometriosis patients by investigating the effect of these treatments on both WT1 and FKBP5 protein expression.

Immunoblot analysis was firstly carried out on ESCs isolated from fertile patients, where protein isolated from an endometriosis patient was also included to allow a direct comparison. It was observed that *in vitro* decidualization resulted in a significant increase in the protein expression of WT1 in the fertile patients ($p \leq 0.01$) (figure 5.30A and B).

This increase in expression was further enhanced on the addition of P4, where WT1 was at a higher expression level in cells treated with cAMP + P4 compared to cells treated with cAMP only ($p \leq 0.05$). Interestingly, co-treatment with cAMP + F also resulted in high levels of WT1 protein expression being present compared to untreated cells ($p \leq 0.01$). However, on the addition of P4 in combination with cAMP + F, there was a dramatic decrease in WT1 levels in the fertile patients compared to cAMP + F only ($p \leq 0.01$). Additionally, treatment with P4 also resulted in a small but significant increase in WT1 expression.

In comparison, even though it was found that treatment of fertile ESCs with cAMP and cAMP + P4 resulted in a significant induction of FKBP5 protein expression compared to the control cells ($p \leq 0.01$) (figure 5.30A and C), there were no differences in the expression of FKBP5 observed between these two treatments. Additionally, no differences in FKBP5

protein expression were observed between cells treated with cAMP + F and those treated with cAMP + F + P4, unlike that observed in WT1 immunoblots.

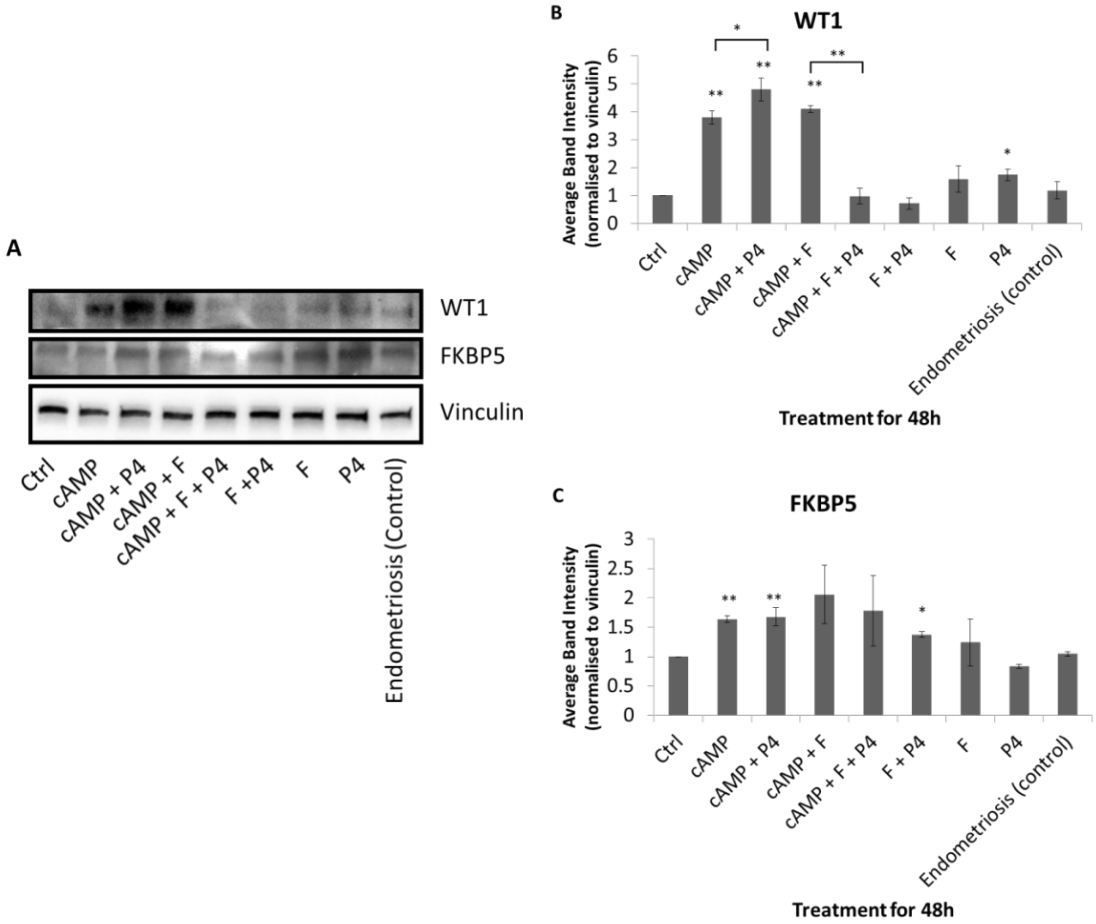


Figure 5.30 The effect of cortisol (F) and progesterone (P4) exposure on the expression of WT1 and FKBP5 during decidual transformation of ESCs isolated from fertile patients. A) Representative images show the western blot analysis of WT1 (52 kDa), FKBP5 (51 kDa) and vinculin (124 kDa) protein levels in ESCs isolated from fertile patients (n=5) after 48h treatment with either cAMP, cAMP + P4, cAMP + F, cAMP + F + P4, F + P4, P4 or F alone compared to the untreated control. B-F) The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the house keeper protein; vinculin. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *p≤0.05, **p≤0.01 and ***p≤0.001 are considered significant.

Lastly, immunoblots were also carried out on protein extracted from ESCs isolated from endometriosis patients, where protein isolated from a fertile patient was also included on the immunoblots to again allow for a direct comparison. As observed in the fertile patients, it was demonstrated that cAMP treatment resulted in a significant induction of WT1 expression compared to untreated cells (p≤0.05) (figure 5.31A and B). However, no enhancement in WT1 expression was observed on the addition of P4 in combination with cAMP. Interestingly, it was found that cAMP + F treatment resulted in significantly lower levels of WT1 expression compared to cells treated with cAMP only (p≤0.05).

In comparison to co-treatment with cAMP + F only, the addition of P4 resulted in a significant decrease in the expression of WT1 compared to cells treated with cAMP + F only ($p \leq 0.05$), which was also observed in the fertile patients. Unlike fertile patients, immunoblot analysis also revealed that co-treatment with F + P4 resulted in a significant decrease in the levels of WT1 compared to controls cells ($p \leq 0.01$).

In ESCs isolated from endometriosis patients, it was found that there was no induction of FKBP5 expression when cells were treated with either cAMP or cAMP + P4 (figure 5.31A and C). However co-treatment with cAMP, F + P4 did result in a significant increase in FKBP5 protein expression compared to untreated control cells ($p \leq 0.05$). It is also important to note that all FKBP5 expression levels regardless of treatment were lower than the basal expression levels of FKBP5 in ESCs isolated from fertile patients.

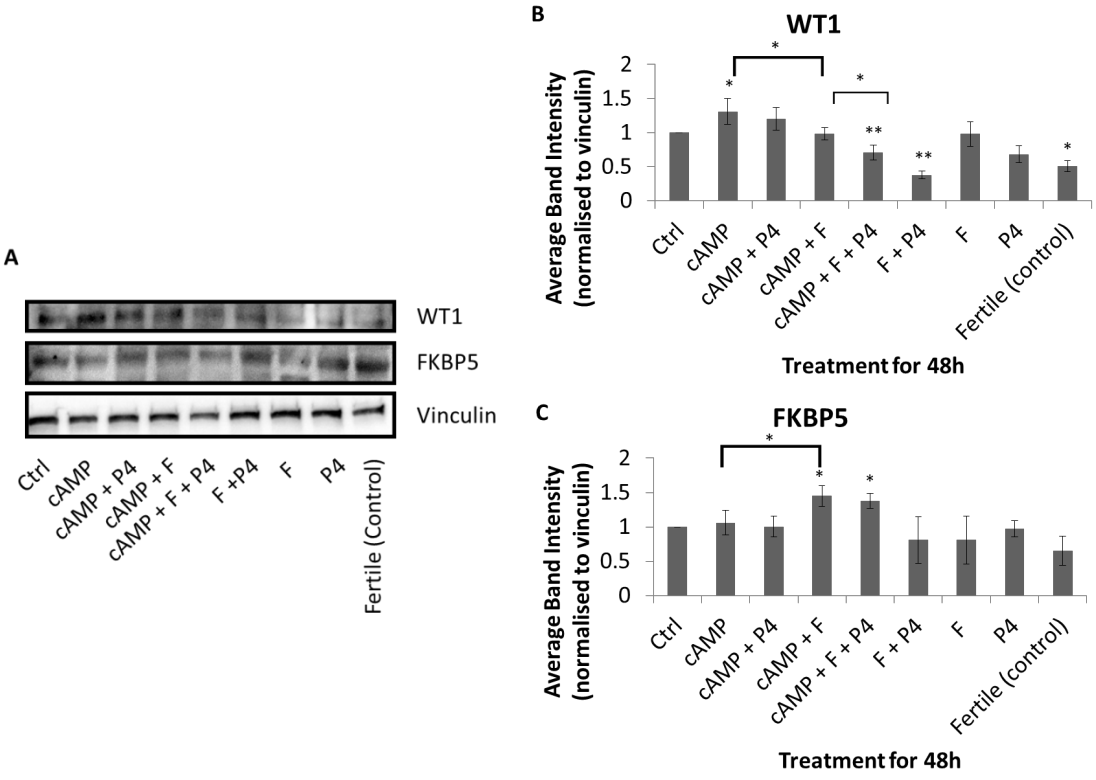


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Figure 5.31 The effect of cortisol (F) and progesterone (P4) exposure on the expression of WT1 and FKBP5 during decidual transformation of ESCs isolated from endometriosis patients. A) Representative images show the western blot analysis of WT1 (52 kDa), FKBP5 (51 kDa) and vinculin (124 kDa) protein levels in ESCs isolated from fertile patients (n=6) after 48h treatment with either cAMP, cAMP + P4, cAMP + F, cAMP + F + P4, F + P4, P4 or F alone compared to the untreated control. B-F) The intensity of the immunoblot bands were measured quantitatively using Image Lab software and normalised to the house keeper protein; vinculin. Values are average ± SD. Data was analysed using an ANOVA test, followed by a student's t-test. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ are considered significant.

5.2.6 Preliminary results showing the effect of the GR signalling pathway in endometrial stromal cells isolated from patients suffering from unexplained infertility (UI)

In addition to the infertile pathologies studied in this chapter, preliminary work was conducted to investigate the effect of GR signalling pathway activation in the stromal cells isolated from patients suffering from unexplained infertility (UI). As previous IHC work in chapter 3 suggests that there is a lower expression of GR in both the glandular and stromal region compared to fertile patients during the proliferative phase of the menstrual cycle (figure 3.10 and figure 3.11). And an increase in GR expression in the stromal region only compared to fertile patients during the secretory phase of the cycle (figure 3.13). In addition to GR expression, a higher concentration of cortisone (compound E) was also observed in patients suffering from UI compared to fertile patients during the proliferative phase of the cycle (figure 3.15) and higher levels of cortisol during the secretory phase of the cycle (figure 3.18). In addition to IHC and ELISA data, whole biopsies were also used to isolate mRNA and protein, where qPCR and western blot analysis respectively revealed an altered expression of both GR and 11β-HSD (figure 3.22 – figure 3.25).

For this study, ESCs isolated from females suffering from UI were isolated and cultured *in vitro* and were treated initially with either cAMP, cAMP + F or F alone and compared to both ESCs isolated from UI patients which were untreated and also to ESCs isolated from fertile patients. After protein extraction, immunoblot analysis was carried out.

Initial results revealed that ESCs isolated from UI patients have a reduced expression of both GR isoforms when treated with either cAMP + F or F alone compared to untreated control cells (figure 5.32A). Interestingly, *in vitro* decidualization seems to result in a loss of the GRβ isoform expression and a reduction in GRα levels. Treatment with F also seemed to have a stimulatory effect on 11β-HSD1 expression compared to control cells. Whereas no significant differences in 11β-HSD2 expression were observed in all treatment groups studied. Furthermore, exposure of ESCs isolated from UI patients to cAMP, cAMP + F or F alone did not have any significant effect on WT1 expression levels compared to untreated cells (figure 5.32B). On the other hand, treatment of the ESCs with cAMP + F or F alone resulted in a significantly enhanced expression of FKBP5 at the protein level (figure 5.32B). Immunoblot analysis was also carried out for PRL, where it was found that exposure to the decidualization stimulus; cAMP, resulted in an increase in the levels of PRL present in the cells. However, treatment with cAMP + F resulted in a decrease in the levels of PRL compared to cells exposed to cAMP and also compared to the control cells. Exposure to F alone resulted in comparable levels of PRL to the control cells (figure 5.32B).

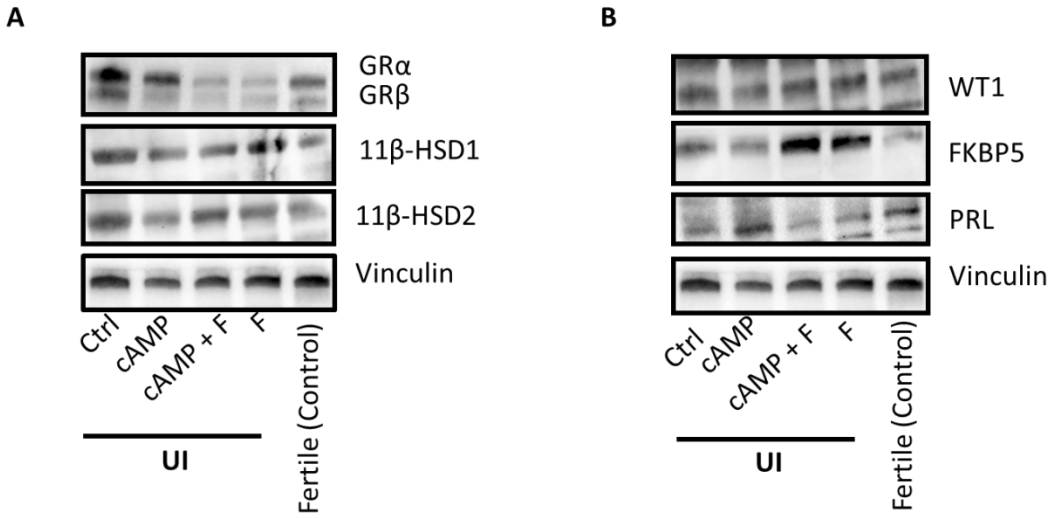

Figure 5.32 The effect of cortisol (F) on key proteins involved in the GR signalling pathway (A) and on the expression of WT1, FKBP5 and PRL (B) during decidual transformation in ESCs isolated from infertile patients suffering from UI. Representative images show the western blot analysis of GRα (95kDa), GRβ (90kDa), 11βHSD1 (32 kDa), 11βHSD2 (40 kDa), WT1 (52 kDa), FKBP5 (51 kDa), PRL (27 kDa) and vinculin (124 kDa) protein levels in ESCs isolated from UI patients (n=1) after 48h treatment with cAMP, cAMP and F or F alone and compared to the untreated control. Untreated ESCs isolated from a fertile patient was also included as a reference for basal levels.

However, it is important to note that this is currently preliminary results due to only one patient suffering from UI taking part in this *in vitro* work. Therefore, further experiments need to be conducted to confirm these results.

5.3 Discussion

This chapter investigates the role of the GC and GR signalling pathway within the human endometrium of infertile patients. Furthermore, this chapter aimed to elucidate whether alterations in the GR signalling pathway may be altering the expression of key players involved in decidualization and therefore the successful establishment and maintenance of pregnancy within infertile patients. This was achieved by determining the effect of cortisol and cortisone exposure on ESCs isolated from infertile patients, either suffering from anovulatory PCOS (anPCOS), ovulatory PCOS (ovPCOS) and endometriosis and compared to fertile patients, during in vitro decidual transformation. Preliminary work was also conducted on ESCs isolated from patients suffering from UI. Additionally, this chapter also aimed to determine whether there was any degree of crosstalk between the GR signalling

pathway and other sex steroid hormone signalling pathways, particularly the AR and PR pathway. These signalling pathways were chosen due to potential dysregulation in the endometrium of infertile patients and due to apparent similarities with GR in terms of structure, DNA binding and recruitment of co-activators (Claessens, Joniau and Helsen, 2017). Studying the potential crosstalk between these pathways was accomplished by co-treating ESCs isolated from infertile patients in the presence and absence of cortisol and androgens (PCOS) or progesterone (endometriosis) during decidual transformation, to monitor the cell morphology, expression of key markers of decidualization and potential GR transcription targets.

Results in this chapter demonstrate that ESCs isolated from infertile patients express both GR isoforms in the stromal compartment of the fertile endometrium (figure 5.1 and 5.2), which is in agreement with chapter 3 IHC findings showing that during the secretory phase of the menstrual cycle, infertile patients display an enhanced expression of endometrial GR compared to fertile patients (figure 3.13). It is important to note that the endometrial biopsies isolated from anPCOS patients are not in the secretory phase of the menstrual cycle, and instead are in the proliferative phase. Immunoblot and qPCR analysis also revealed that GR expression is altered in the stromal compartment in these patients (figure 5.1 and 5.2). This provides further evidence for the hypothesis that infiltrating immune cells, such as macrophages and uNK cells, which are known to express GR are significantly altering the GR expression levels found in protein isolated from whole tissue biopsies, as described previously. Therefore, independent of the pathology, cell type is crucial for the investigation of endometrial GR expression. Furthermore, the effect of infiltrating immune cells may be further enhanced in infertile conditions, especially endometriosis, due to the endometrium favouring a pro-inflammatory environment (Monsivais et al, 2012). Interestingly, IHC analysis presented in chapter 3 was not able to distinguish between the two primary GR isoforms; α and β. However, due to differences in their molecular weight – immunoblot analysis is able to provide a clear resolution between the two protein isoforms. It was observed that the less dominant GRβ isoform has a reduced endometrial expression within the stromal compartment in the endometrium of infertile patients (figure 5.1). This supports results obtained using whole endometrial biopsies to identify the expression of GR, where a significant decrease in GRβ was observed (figure 3.25). GRβ is known to act as a dominant inhibitor of GRα expression and therefore can inhibit the GR signalling pathway (Bamberger et al, 1995). However the infertile endometrium exhibits up-regulation of GRα with a simultaneous down-regulation of GRβ, favouring the activation of the GR signalling pathway within these cells at the basal level. Of interest, is that the endometrial expression

of both GR isoforms in ESCs isolated from endometriosis and UI patients was found not to be significantly altered compared to fertile patients (figure 5.1).

In addition to GR, investigations were made to identify the expression levels of 11β-HSD1 and 11β-HSD2 in the infertile endometrial stromal compartment. It was identified that infertile patients display an increased expression of 11β-HSD1 at the mRNA level and protein level (with the exception of ovPCOS patients) favouring an increased local bio-availability of cortisol, assuming the enzymatic activity is fully functional. The results obtained for anPCOS patients are in agreement with previous studies, which have reported an over-expression of 11β-HSD1 in the adipose tissue and granulosa cells of PCOS patients, which may contribute to the high levels of cortisol present in the follicular fluid in these patients (Zhu et al, 2016). These results contradict results presented in chapter 3 using whole tissue biopsies which suggest that the expression of 11β-HSD1 is decreased in the endometrium of endometriosis patients, further supporting the hypothesis that cell type is critical in these studies. However, it is worth mentioning that all grades of endometriosis disease were grouped as a single cohort in this study and hence it is difficult to correlate whole biopsy data to cell compartment expression within this group. The endometrial stromal expression levels of 11β-HSD2 did not show any significant difference in the ESC expression in infertile patients, further supporting the increased cortisol levels observed in these patients during the secretory phase of the menstrual cycle (chapter 3.18).

These investigations into the basal endometrial expression levels of GR and 11β-HSD in the stromal region of infertile endometrium identified a potential altered GR signalling pathway which was further investigated. Therefore, it was decided to repeat the experiments conducted in chapter 4 in order to investigate the effect of the stress hormones during decidual transformation of ESCs isolated from infertile patients suffering from ovPCOS, anPCOS or endometriosis. In addition, the potential crosstalk of the GR signalling pathway and the sex steroid hormone signalling pathway was also investigated. .

It was found using ESCs isolated from anPCOS patients, that interestingly GC exposure, whether it is in the active or inactive form i.e. cortisol or cortisone, did not provoke major alterations in the measurements of in vitro decidual transformation, namely morphology and decidual marker expression (figure 5.3 and 5.4 or figure 5.11 and 5.12). Regardless of GC exposure, decidual transformation was already defective in these patients.

It was found that in vitro decidualization of ESCs isolated from anPCOS patients didn't alter GRα isoform expression in response to cortisol or cortisone treatment, instead a significant reduction in GRβ endometrial expression was observed in response to cortisol, which has yet

to be reported to date (figure 5.6). In contrast, exposure to the decidualization stimulus in the presence of the stress hormone, cortisol resulted in a simultaneous down-regulation of endometrial GRα expression and an up-regulation of GRβ expression. However, this effect was not reciprocated in response to cortisone treatment. Interestingly, cortisol treatment alone was able to significantly reduce GRα activity, suggesting this pathway may be important in the endometrium of anPCOS patents subject to stress. Using ESCs isolated from anPCOS patients, no significant alterations in 11β-HSD1 expression were observed in response to either cortisol or cortisone during decidual transformation; however an up-regulation of the type 2 isoform was exhibited in these cells (figure 5.6 and figure 5.14). This could potentially be considered a feedback mechanism provoked by the high circulating cortisol levels observed in these patients, which could induce expression of 11β-HSD2 to counteract cortisol effects. Interestingly, it was observed in chapter 4, that 11β-HSD1 seems to be a marker of decidual transformation which is significantly induced during successful decidual transformation in the fertile patients (figure 4.6 and 4.7). Therefore the lack of 11β-HSD1 induction in anPCOS patients also indicates that decidual transformation is defective or delayed in these patients which may be due to the fact that PCOS patients suffer from deficiencies in their cAMP response (Gonzalez et al, 2012). In support of this, it was observed in anPCOS samples that the expression of the GR target WT1, which is also a key regulator of decidual transformation is not only reduced in response to the decidualization stimulus, but is further down-regulated in response to cortisol exposure during decidualization of the endometrial stromal compartment (figure 5.9 A and C). This is in accordance with previous studies conducted by the RBGO group, which also observed that PCOS patients have dysregulated WT1 expression levels during decidual transformation (Gonzalez et al, 2012). However, cortisone was not able to induce the same effect on WT1 expression, indicating that it is the active ligand that is required to activate the GR signalling pathway and allow GR to alter the transcription of WT1. In contrast, FKBP5 expression was instead up-regulated in response to cortisol and cortisone exposure during decidual transformation of anPCOS stromal cells (figure 5.9A and D). This suggests that FKBP5 may not play a significant role in defective decidualization in anPCOS patients, but may be instead crucial in co-chaperoning GR within the ESCs (Yeo et al, 2017). Cortisol exposure during decidual transformation had no effect on the endometrial expression levels of AR or PRL in anPCOS patients; however the exposure to cortisone during decidual transformation resulted in a significant decrease in the expression of endometrial PRL expression (figure 5.17). This suggests that GR ligands may not only interfere with early decidualization signalling pathways like WT1, but also other pathways involved in later stages of decidua transformation.

Further experiments to identify potential crosstalk mechanisms between the GR and AR signalling pathway in anPCOS patients were also conducted using the androgen; DHT and cortisol. Cortisol was chosen as the primary GC due to the ELISA results presented in chapter 3, which demonstrate that PCOS patients have a significantly higher serum levels of cortisol during the secretory phase of the cycle (figure 3.18) compared to cortisone and is therefore more clinically relevant. It was found that the presence of androgens (DHT) in combination with cortisol during decidual transformation did not have a significant effect on ESC morphology or ELISA levels of decidual markers compared to cells exposed to androgens when decidualization was measured for 48h (figure 5.18). The effect of androgen and cortisol exposure during decidual transformation on the endometrial expression levels of potential GR targets identified that the combined exposure of androgens and cortisol during decidual transformation resulted in endometrial expression levels of AR similar to that observed with cAMP + DHT only (figure 5.22). The addition of androgens during decidual transformation had no effect on GRα expression and interestingly, it was also found that GR expression levels were not affected significantly when cells were treated with cortisol in combination with androgens during decidual transformation compared to cells treated with cortisol only during this process, suggesting that there is a not a cumulative effect of androgens and cortisol on either AR or GRα expression (figure 5.22). On the other hand, androgen exposure has a significant effect on GRβ exposure during in vitro decidualization in the presence and absence of cortisol (figure 5.22 A and D). This suggests a novel interaction between the AR and GR signalling pathways in the endometrium of anPCOS patients. Furthermore, androgen exposure during decidual transformation also results in the inhibition of 11β-HSD expression. Of particular interest to this study is that there is a cumulative effect of androgen and cortisol exposure on the reduction of endometrial WT1 and FKBP5 expression in anPCOS patients during decidual transformation, where co-treatment with the in vitro decidual transformation, cortisol and androgens resulted in practically a complete loss of WT1 expression (figure 5.25). This indicates that anPCOS patients suffering from androgen excess who are exposed to chronic stress levels may suffer from enhanced defects in decidual transformation. Further experiments beyond 48h could further elucidate the effects of GR on WT1 signalling pathway, which is known to be a master regulator of decidualization (Gonzalez et al, 2012).

Similarly, to that seen in anPCOS, it was found that in ovPCOS, cortisol or cortisone exposure during decidual transformation, did not alter the target markers of decidualization, including cell morphology and decidual markers (figure 5.3 and 5.4 or figure 5.11 and 5.12). However, contrary to anPCOS, ESC from ovPCOS patients exhibits an increase in GRα expression with a concomitant decrease in GRβ expression after decidual stimulation (figure

5.6). The addition of the stress hormone cortisol resulted in a switch of GR isoform expression profiles within the stromal region in ovPCOS patients where the GRα expression levels decrease and GRβ levels increase. Whereas the addition of cortisone, did not result in this switch of endometrial expression of the GR isoforms (figure 5.14). No significant alterations in the endometrial stromal expression levels of either 11β-HSD isoforms were observed in ovPCOS patients in response to cortisol or cortisone exposure during decidual transformation (figure 5.6 or 5.14). Investigations into the role of cortisol exposure on the modulation of key master regulators of decidualization identified that, similar to anPCOS patients, ESCs isolated from ovPCOS patients also displayed a decrease in WT1 expression in response to cortisol exposure during decidual transformation, which was not reciprocated if cortisol was replaced for cortisone. In addition, the same effects reported for anPCOS were also observed for ovPCOS patients in FKBP5 and dPRL (figure 5.9). This indicates that it is the active hormone cortisol, which is crucial in modulating the expression of WT1 in the endometrial stromal region in PCOS patients. More importantly, the differential effect of cortisol in GR isoform expression in anPCOS and ovPCOS needs to be further investigated and suggest that different signalling pathways are altered in these patients during decidual transformation.

Experiments to identify potential crosstalk mechanisms between the GR and AR signalling pathway in ovPCOS patients were also conducted using the androgen; DHT and cortisol. It was identified that the combination of androgens and cortisol resulted in significant reductions in both the expression of GR isoforms and the expression key markers of decidualization; WT1 and FKBP5 (figure 5.24 and 5.25), as was also observed in anPCOS patients. This suggests a degree of crosstalk between GR and AR within the endometrium, which is supported by previous studies which have identified that AR and GR are able to modulate each other's transcriptional activity when co-expressed within tissue (Chen et al, 1997). Conversely, in ESCs isolated from ovPCOS patients, it was found that DHT stimulations, alone or in combination with cAMP or cAMP and cortisol, significantly induced the expression of the 11β-HSD2 enzyme (Figure 5.21).

It was not only the PCOS cohorts which were confined to the effects of androgens and cortisol during decidual transformation. In the fertile endometrium, it was found that androgen stimulations in combination with cortisol during decidual transformation also resulted in significant up-regulation of key proteins involved in the GR signalling pathway (GRα and 11β-HSD) (figure 5.20) with the simultaneous down-regulation of the morphological and molecular decidual markers (figure 5.23 and 5.18). These results confirm the importance of exploring additional signalling pathways in these patient cohorts under

investigation in relation to GR activation, given that in vitro decidualization in the presence of androgens and cortisol results in the variable expression levels of the GR isoforms dependent on reproductive pathology. It must be noted that even though it is known that an excess level of androgens present locally within the endometrium can prove detrimental to fertility and the menstrual cycle, it is however known that androgens at low levels are also important within the endometrium of fertile patients, particularly during the decidual response (Cloke et al., 2008). Genome-wide expression profiles revealed that it is not only the PR and its ligand; progesterone, which has a crucial role in decidualization but also AR and androgens, where both signalling pathways regulate the expression of distinct decidual gene networks (Cloke et al, 2008). It is known that the ESCs isolated from fertile patients also express functional AR which is activated by the presence of androgens (Maybin and Critchley, 2012) (figure 5.21), in addition to the aberrant expression witnessed in PCOS patients.

The results presented in this chapter, clearly demonstrate that the relevant levels of the sex steroid hormones and the stress hormones are critical for the successful transformation of the endometrium in preparation for implantation. Any alterations in the homeostasis of the steroid hormones, whether that is excess androgen levels due to the presence of PCOS or excess cortisol levels due to chronic stress exposure or even a combination of the two, can ultimately result in a detrimental impact on the fine balance of the signalling pathways within the human endometrium leading to sub-fertile or infertile phenotypes. Therefore, PCOS patients who also suffer psychologically due to the clinical manifestations of the syndrome may have a significantly defective decidual response pathway due to the cumulative effect of excess androgens and high levels of cortisol present, which is in agreement with epidemiological evidence suggesting the psychological state of PCOS patients may be related to their infertility status (Brady, Mousa and Mousa, 2009).

In addition to PCOS, it was found using ESCs isolated from endometriosis patients that cortisol exposure during decidual transformation results in a delayed decidual transformation of the ESCs, in terms of their characteristic fibroblastic phenotype, to the rounded epithelial-like phenotype required for decidual transformation compared to cells exposed to the decidual stimulus only (figure 5.3). This delay in decidual transformation was further supported by the down-regulation of WT1 and PRL expression in response to cortisol exposure during decidualization (figure 5.10). Interestingly, decidual transformation resulted in the induction of GRα, 11β-HSD1 and 11β-HSD2 expression, with the simultaneous down-regulation of GRβ (figure 5.7); however, these pronounced effects were reversed with the addition of F during decidual transformation of ESCs isolated from

endometriosis patients (excluding 11β-HSD2). Therefore, the pattern of endometrial GRβ and 11β-HSD1 protein expression differs in endometriosis patients compared to other infertile pathologies. The induction of GR expression in response to decidualization and the increased endometrial basal levels of GR is supported by research conducted by Monsivais and colleagues, who demonstrated that the serum and glucocorticoid regulated kinase 1 (SGK1) is over-expressed in the stromal compartment of endometriosis patients, where the aberrant expression of this protein is related to recurrent pregnancy loss and infertility (Monsivais et al, 2016). SGK1 is able to interact with the active GR signalling pathway in addition to other steroid hormone receptors within the endometrium and is highly expressed during decidual transformation (Walsh et al, 2012; Monsivais et al, 2016). Furthermore, endometriotic stromal cells isolated from eutopic endometrium have also been found to synthesise and secrete high levels of cortisol locally, this supports data presented in this chapter as this may be due to an increase in the activity of the 11β-HSD1 enzyme and the GR signalling pathway in these patients, which was observed in the results presented in this chapter. These conditions may develop due to the pro-inflammatory environment in the endometriotic lesions (Monsivais et al, 2012). This hypothesis is supported by the fact that other inflammatory disorders, such as rheumatoid arthritis (RA) and Crohn's disease also have a high expression of GR and 11β-HSD1 (Neeck et al, 2002; Stegk et al, 2009). Therefore, inflammation may play a significant role in the GR signalling pathway, particularly within the human endometrium.

Endometrial stromal PR also followed the same pattern of expression as GRα in response to decidual transformation alone and with the addition of cortisol (figure 5.10). It is also important to note, that replacing cortisol for cortisone in these 48h experiments also resulted in similar effects on WT1 and PRL expression and the relative expression levels of the 11β-HSD isoforms compared to cortisol exposure, but the effects were less pronounced (figure 5.17 and figure 5.15). Despite this, the clear switch of endometrial GR isoform expression seen in ESCs isolated from endometriosis patients in response to decidualization stimulation and cortisol exposure was not observed in the presence and absence of cortisone (figure 5.15).

In addition to androgens, it is known that progesterone (P4) is a key hormone involved in regulating the female reproductive system and plays a major role in the decidualization and implantation processes and elevated circulating levels of P4 maintain the decidual response within the endometrium during pregnancy and is therefore crucial for the successful development and maintenance of pregnancy. The effects of P4 are mediated through the progesterone receptor (PR) (Patel et al, 2015). As the GR signalling pathway may also play a

role in the decidualization process it was decided to explore the potential crosstalk between the PR and GR signalling pathways. Endometriosis patients were recruited for this section and compared to fertile patients due to the hypothesis that the development and pathophysiology of the condition may be related to progesterone resistance (Patel et al, 2017; Moberg et al, 2015) and aberrant PR signalling pathways may also be present in some patients suffering from endometriosis (Patel et al, 2015).

Results presented in this chapter identify that the presence of cortisol (F) and P4 results in similar levels of decidual transformation observed when cells are treated F only during decidual transformation in terms of cell morphology and the expression of the secreted decidual markers in both endometriosis and fertile patients (figure 5.26 and 5.27). Interestingly, it was found that the combined effect of P4 and cortisol during decidual transformation resulted in the induction of PR expression, the reduction of GRα and β expression and an increase in the expression of 11β-HSD1 in the fertile patients (figure 5.28). However it is important to note, that the levels of PR expression were not as high as that observed for fertile cells treated with the decidualization stimulus in combination with cortisol, suggesting that cortisol is able to modulate the expression of PR in the stromal compartment, which is supported by the ChIP data presented in chapter 4 (figure 4.29). Furthermore, it is known that P4 is able to modulate uNK cells via the GR signalling pathway (Guo et al, 2012) and the GR signalling pathway interacts with PR to produce P4-like effects in breast cancer cells (Leo et al, 2004). 11β-HSD1 seems to be a potential marker for the decidual response, which is supported by previous research (Takano, 2007). As such, co-treatment with cAMP + P4, which are both used as well-characterised initiators of in vitro decidualization, resulted in a dramatic rise in the enzyme expression, which was reduced with the addition of F (figure 5.28). Interestingly, in endometriosis patients – treatment of the ESCs with F during decidual transformation did not result in a dramatic increase in PR expression as seen in the fertile patients, suggesting that cortisol is not able to modulate PR expression to the same extent in endometriosis patients (figure 5.29). Alterations in GR and 11β-HSD isoform expression were also observed in endometriosis patients in response to P4 and cortisol exposure during decidual transformation, where immunoblot analysis revealed that there is no down-regulation of GRα or β and no simultaneous up-regulation of 11β-HSD1 (figure 5.29). Interestingly, co-treatment of ESCs isolated from fertile patients with P4 and cortisol during decidual transformation resulted in a clear reduction of WT1 expression (figure 5.30) which was also displayed in endometriosis patients (figure 5.31). This provides further supporting evidence that cortisol has a significant detrimental effect on decidual transformation in both fertile and infertile patients.

Conversely, FKBP5 largely remains unaffected in both fertile and endometriosis patients in response to cortisol and P4 stimulations (figure 5.30 and 5.31).

It is known that the previously mentioned co-chaperone of GR; FKBP4 has a reduced expression in the endometrium of endometriosis patients compared to fertile patients, particularly during decidual transformation (Yang et al, 2012). This suggests that FKBP4 and the GR signalling pathway may play a role in decidualization of the ESCs, which is a process known to be impaired in some endometriosis patients. Additionally, the dysregulation of FKBP4 expression may also be having an effect on other steroid hormone receptors, such as PR – which may be contributing to the progesterone resistance sometimes exhibited in endometriosis patients and therefore may have an impact on the development and progression of this disease (Guy, Garcia and Cox, 2015; Yang et al, 2012). Therefore, even though FKBP5 expression remains largely unaffected, the investigation of FKBP4 expression may shed more light on the role of GR signalling within the decidua in these patients.

Preliminary experiments were also conducted on ESCs isolated from UI patients, which demonstrated that the GR expression, its targets and signalling pathway is altered in the stromal compartment of these patients in response to decidual transformation in the presence and absence of the stress hormone; cortisol (figure 5.32). Therefore, indicating that stress exposure and the GR signalling pathway may be having a significant effect on the endometrium of these patients. This preliminary data is supported by epidemiological evidence of fertile patients undergoing periods of UI, which may be in part due to stress exposure (Lynch et al, 2014) and patients suffering from UI exhibit higher levels of stress compared to fertile patients (Harrison, O'Moore and O'Moore, 1986). Nonetheless, a study by Donarelli and colleagues reported no relationship between perceived stress and ovarian response in females suffering from UI (Donarelli et al, 2016). Thus indicating the need for extensive molecular analysis of the GR signalling pathway in UI patients.

Lastly, initial qPCR analysis was carried out to identify whether infertile patients exhibit altered expression levels of MR and/or GR in the endometrial stromal compartment at the mRNA level (figure 5.8). Decidualization is associated with a genetic switch of the corticosteroid receptors, where GR is down-regulated and MR is induced as presented in chapter 4 (figure 4.8). In stark contrast, this switch of receptor dominance is not observed in infertile patients, where GR expression remains high and MR expression remains low, suggesting a potential role for MR signalling in addition to GR in the defective decidual response observed in the infertile patients under investigation in this study. Further work is

also needed to identify GR and also MR dependent genes in differentiating ESCs (Cloke, 2008; Kuroda et al, 2013).

The results presented in this chapter support the work conducted by Whirledge and colleagues (2015), who have established a crucial role for GR in the mouse endometrium. GR KO mice were found to have defective decidualization and implantation (Whirledge et al, 2015). Our results demonstrate that GR and its consequent signalling pathway could play a significant role during decidual transformation of human ESCs, similar to that seen in the GR KO mice studies. Therefore the subsequent knock-out of GR in ESCs is essential to allow us to establish if GR is having a direct effect on this key process. Furthermore, the GR signalling pathway in combination with the well-characterised sex hormone signalling pathway and their apparent crosstalk may play a crucial role in maintaining normal endometrial homeostasis, ensuring successful decidual transformation, which is essential for establishing and supporting pregnancy.

CHAPTER 6

General Discussion

and

Conclusions

6.1 Thesis summary and discussion

Given the extensive body of evidence within the literature which implicates stress in the propagation of infertility, the results presented in this thesis served to elucidate a potential relationship between the activation of the GR signalling pathway, endometrial pathology and decidual transformation in both fertile and infertile patients.

The principal aim of this thesis is to present novel evidence for the expression of GCs and its nuclear receptor, GR in the endometrium of fertile and infertile patients and to relate these observations to key features of common reproductive endocrinological pathologies namely PCOS, endometriosis and UI. Secondary to this, was to establish the effect of the GR signalling pathway activation in the human endometrium during the process of decidual transformation.

Our results demonstrate that GR isoforms are expressed during the menstrual cycle in the fertile endometrium supporting a role for GR signalling pathway in reproductive function. In fact, both GR isoforms under investigation are expressed in the fertile endometrium throughout the menstrual cycle (figure 3.9, Bamberger et al., 2001) and therefore may play a critical role in maintaining the homeostasis of reproductive function. This is supported by previous studies using GR KO mice, which were found to have defects in the endometrium leading to impaired decidualization and implantation (Whirledge et al, 2015). IHC analysis revealed that endometrial GR expression significantly decreases in the glandular and stromal region during the secretory phase of the menstrual cycle in fertile patients (figure 3.3). Conversely, this study identified that the expression of both GR isoforms within the uterus of fertile patients increase during the secretory phase of the menstrual cycle (figure 3.9). This effect is contradictory to the IHC results obtained previously in this study, but is probably due to a rise of infiltrating immune cells, which are involved in processes in the endometrium during early pregnancy, including uterine remodelling and the induction of the expression of epithelial glycoproteins required for embryo attachment and implantation (Brown et al, 2014; Jasper et al, 2011). These infiltrating immune cells are known to express GRα in particular, and therefore the presence of these cells will also be providing us with positive expression of GR. This highlights the importance of the endometrial cell type when investigating the GR signalling pathway. In this context, the top biological functions identified by GO analysis of the genes regulated in uterine GR KO mice included chemotaxis, inflammatory response, immune response, and immune cell migration, which are functions described for immune cells and for decidua tissue in the human uterus. Data presented in this study, demonstrates that although the expression of GR protein decreases significantly from the proliferative to the secretory phase when measured by IHC, *in vitro*

decidualization resulted in the increase of endometrial GRβ isoform expression with either no changes in GRα expression in regards to protein level (figure 4.6) or reductions in GRα expression at the mRNA level (figure 4.7). These results thus support previous microarray analysis, which identified that the programming required to successfully induce decidualization are deficient in GR KO mice (Whirledge et al, 2015), indicating that the GR signalling pathway is crucial during the decidual response and a fine-balance of the GR receptors is required for endometrial decidual transformation to take place.

Interestingly, in addition to endometrial GR expression it was also identified in the fertile patients that their circulating cortisol levels decreased significantly during the secretory phase of the menstrual cycle compared to the proliferative phase (figure 3.4) Furthermore, these patients exhibited a concomitant increase in circulating cortisone levels (figure 3.5). The reduction in systemic cortisol levels supports the potential attenuation of the GRα isoform during the secretory phase of the menstrual cycle, as cortisol binds with a higher affinity to GRα, therefore reductions in cortisol concentration could result in reductions in GRα expression (Lippman et al, 1974). Furthermore, the systemically high levels of cortisol observed during the proliferative phase of the menstrual cycle may result in a negative feedback loop of the GR signalling pathway in response to pathway activation, which protects the tissues from the local exposure of the excess cortisol and prevents tissues entering a hypercortisolemic states. This GC-mediated down-regulation of GR may be due to GR inhibiting its own transcription by interrupting with the transcriptional activation of the NR3C1 gene via Activator protein 1 (AP-1) (Bamberger, Schulte and Chrousos, 1996). In addition to GCs, the effects of other steroid hormones and their signalling pathways are also important and add complexity to the GR signalling pathway within the endometrium. It has been reported that GR through protein-protein binding can tether to other TFs present within the cell, independent of DNA binding at the promoter region of target genes and interfere with their mechanism of action (Coutinho and Chapman, 2011). In fact, GR has been shown to interact with ER, for example in breast and endometrial cancer cells (Karmakar et al, 2013; Vahrenkamp et al, 2018). Interestingly, in the GR KO mice, GR ablation does not impair estrogen responsiveness, but attenuates the endometrial response to PR signalling (Whirledge et al, 2015). In agreement, ChIP data shown in chapter 4 indicates that in the presence of GCs, GR is able to significantly bind to the PGR promoter and may potentially regulate its expression (Figure 4.29 and figure 5.28). This suggests that in the endometrium of fertile patients, GR could potentially interact with PR and result in the modulation of this crucial signalling pathway during decidual transformation and help sustain the decidual response.

Additional work presented in this study supports a role for GR and its signalling pathway in endometrial function, results show that ESCs isolated from fertile patients exposed to the stress hormone cortisol and an *in vitro* decidualization stimulus delayed the morphological transformation of the ESCs, further decreased the endometrial stromal expression levels of the GR isoforms and attenuated the expression of key decidual markers (except WT1) (figure 6.1). Thus suggesting that stress exposure may influence reproductive function through impairment of decidualization.

It is now widely accepted that reproductive functions and the regulation of the stress response are systems which are closely regulated in humans (Iwasa et al, 2018). Reproductive functions are suppressed by various kinds of stressors such as infection, psychological burdens, excessive exercise and malnutrition. Once stress levels are reduced, reproductive functions are normally restored. There have been numerous studies suggesting that patients undergoing fertility treatments suffer from increased levels of stress and higher prevalence of depressive symptoms compared to the general population (Demyttenaere et al, 1992; Lakatos et al, 2015; Massey et al, 2014). The molecular mechanisms underlying the impact of stress on female infertility remain to be elucidated. Interestingly, it must be noted that the majority of studies which have been previously published which have investigated the association between stress and infertility are cross-sectional studies of couples actively seeking ARTs, which results in difficulties in identifying the directionality of the association between stress and infertility (Lynch et al, 2014). Furthermore, it is unknown how stress may be affecting the clinical outcomes of fertility treatments, such as IVF. It has been found that psychosocial interventions in couples who are undergoing fertility treatment are effective in reducing psychological distress and can lead to an improvement in clinical pregnancy rates (Cesta et al, 2018). In light of this, the results presented in this study provide compelling evidence of the effect of the GR signalling pathway and stress in the transformation of the human endometrium to support pregnancy.

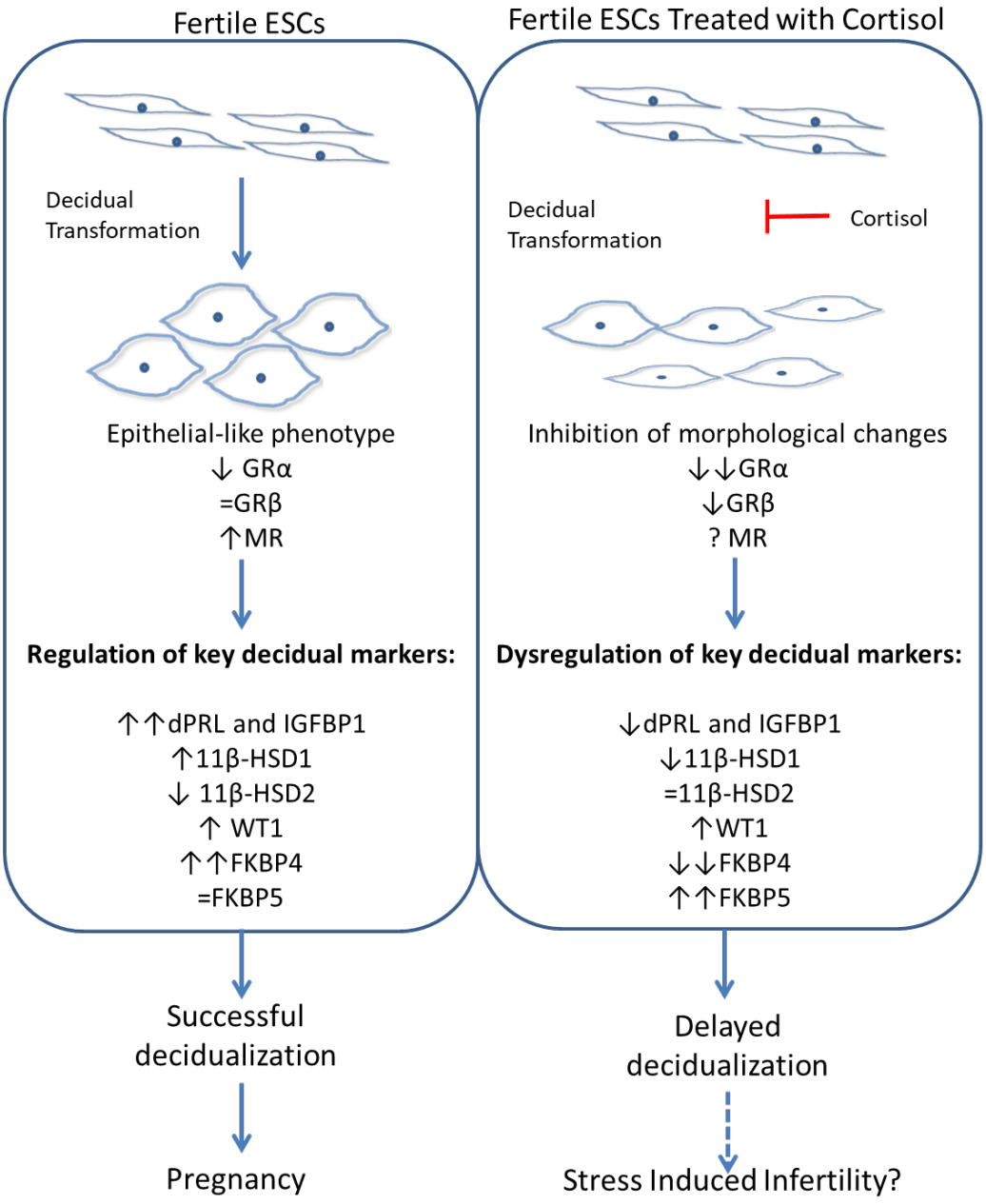

Figure 6.1 The comparison of the *in vitro* decidual response in ESCs isolated from fertile patients compared to ESCs isolated from fertile patients and consequently exposed to the stress hormone cortisol. ESCs isolated from fertile patients who are not exposed to the stress hormone cortisol, successful *in vitro* decidualization is able to take place due to the characteristic morphological changes of the ESCs, induction of key decidual markers and concomitant down-regulation of GRα, which can result in the successful development and maintenance of pregnancy. Whereas fertile patients exposed to the stress hormone cortisol, results in delayed decidualization due to the inhibition of key morphological changes of the ESCs, further decreases of the GR isoforms and the reduction of key decidual markers (except WT1), which can result in stress induced infertility ('↑' = indicates up-regulation, '↓' = indicates down-regulation and more arrows indicate level of up or down regulation dependent on arrow direction, '=' indicates no significant alteration in expression, '?' indicates unknown effect).

In addition, data obtained in this study identified alterations in GR isoform expression, cortisol levels and expression of the 11β-HSD1 enzyme in the endometrium of infertile patients, either suffering from PCOS, UI or endometriosis during the proliferative and secretory phase of the menstrual cycle compared to fertile patients (Table 6.1).

A) Proliferative Phase (vs Fertile)	anPCOS	ovPCOS	Endometriosis	UI
Endometrial GR Levels				
Glandular Compartment	↓	↓	↓	↓
Stromal Compartment	↓	↓	↓	↓
Uterine Levels				
GR	=	=	=	↓ GRα = GRβ
11β-HSD1	↓	↓	↓	=
Systemic Levels				
Serum Cortisol Levels	=	=	=	=

B) Secretory Phase (vs Fertile)	ovPCOS	Endometriosis	UI
Endometrial GR Levels			
Glandular Compartment	=	=	=
Stromal Compartment	↑	↑	↑
Uterine Levels			
GR	↓	= GRα ↓GRβ	↓
11β-HSD1	=	↓	↓
Systemic Levels			
Serum Cortisol Levels	↑	=	↑

Table 6.1 Tables summarising the expression of GR, cortisol and 11β-HSD1 in the infertile endometrium in comparison to the endometrium of fertile patients during the proliferative (A) and secretory (B) phases of the menstrual cycle. This table illustrates the endometrial GR expression levels within the glandular and stromal compartments using IHC analysis, uterine levels of GR and 11β-HSD1 using whole tissue biopsies and consequent qPCR and immunoblot analysis and the systemic levels of cortisol using commercially available ELISAs in both the proliferative and secretory phase of the menstrual cycle. ('↑' = indicates up-regulation, '↓' = indicates down-regulation, '=' indicates no significant alteration in expression). It must be noted that the anPCOS patient cohort were not included in the secretory phase studies, due to them being chronically in the proliferative phase of the menstrual cycle.

Overall, this data suggests that the crucial GR signalling pathway is altered in the infertile endometrium, particularly as increased levels of circulating cortisol are observed in these patients. The WHO now recognises that both males and females suffer from psychological issues when experiencing reproductive health issues, which include infertility with no evidence of disease or infertile pathologies such as PCOS and endometriosis. This is evident

by the fact that patients suffering from PCOS and endometriosis are significantly affected by mental health issues, including depression and higher levels of perceived stress (Conway et al, 2014; Tariverdian et al, 2007).

Moreover, when *in vitro* decidualization was assessed using ESCs isolated from PCOS and endometriosis patients, stark molecular differences were observed in the presence of the stress hormone cortisol. Decidualization has been reported to be delayed in PCOS patients and importantly, the results of this current study further confirm these observations (Figure 6.2; Gonzalez et al., 2012). Interestingly, the GRα isoform is upregulated in decidualized ESCs isolated from PCOS patients, compared to fertile patients. This effect is reversed on the addition of cortisol, but with a concomitant decrease in GRβ levels in anPCOS patients only. This fine-balance of the expression of the GR isoforms seems to be of particular importance during successful decidualization in fertile patients. In addition, cortisol exposure seems to either further or maintain the decrease the expression levels of key decidualization markers; WT1, FKBP5 and 11β-HSD1 in PCOS patients. Results differ depending on whether the PCOS patients are able to ovulate, which highlights the importance of the sub-groups in this patient cohort. The molecular evidence presented implicates cortisol activation of the GR signalling pathway and the consequent decrease of the GR isoforms as having a detrimental effect on the decidual transformation process in the PCOS patients. Interestingly, altered sex steroid hormone production and signalling pathways have already been described previously in the endometrium of PCOS patients. For example, variations in PR signalling in the endometrium of PCOS patients can result in P4 resistance (Li et al, 2014). The PR and P4 are vital for the successful endometrial differentiation of ESCs into decidual cells. Potential crosstalk between the GR signalling pathway and the other sex steroid hormone pathways, particularly PR, was described to potentially modulate the PR signalling pathway and support decidual transformation in fertile patients in this study previously. Alterations in the GR signalling pathway in PCOS patients may result in this crosstalk being impaired, leading to defects in the decidual response observed in response to cortisol exposure. Even though it is well established that P4 and the PR is vital for decidual transformation, the GR signalling pathway may also have a significant level of cross talk with the AR signalling pathway in the endometrium of PCOS patients which may result in defects in decidual transformation. It is known that the AR signalling pathway is able to modulate a distinct gene network in decidual cells related to cytoskeletal re-organisation and cell motility and the presence of regulated low levels of androgens is therefore crucial for the decidual response (Cloke et al, 2008). Aberrant expression and the dysregulation of the AR signalling pathway in addition to high circulating androgen levels is known to impair the decidual response in PCOS patients by

reducing the expression of key decidual master regulators such as WT1 (Gonzalez et al, 2012). In addition to this, GR is able to crosstalk with the AR signalling pathway which has been identified in prostate cancer cells (Arora et al, 2013) and bladder cancer (McBeth et al, 2015), potentially through direct tethering at the AR promoter to GREs in the fertile endometrium as demonstrated in table 4.3 and figure 4.30. Therefore, the dramatically elevated levels of both cortisol and androgens observed in PCOS patients during the secretory phase and decidual transformation may have a detrimental impact on GR and AR crosstalk and may be implicated in delayed decidual responses in the endometrium of PCOS patients.

Infertile endocrine pathologies including PCOS have been associated with a list of psychopathological disturbances and reduced quality of life that suggest that significant alterations of the HPA axis in response to chronic stress may take place (Papalou and Diamanti-Kandarakis, 2017; Brady, Mousa and Mousa, 2009). It is known that those suffering with PCOS are more likely to present with significantly higher stress and anxiety levels and are also more likely to self-harm (Hart and Doherty, 2015). For example, a recent meta-analysis has revealed that PCOS sufferers have a fourfold risk for depressive symptoms compared to the age-matched general population (Papalou and Diamanti-Kandarakis, 2016). The elevated levels of stress in this patient cohort may be due to the presence of the clinical manifestations of this syndrome, which include hirsutism irregular menstruation, infertility, recurrent miscarriages, obesity and the metabolic syndrome, which can adversely affect psychological health by producing body dissatisfaction and low self-esteem (Rotterdam ESHRE Criteria, 2004). Interestingly, a specific PCOS obesity phenotype has been described, which is related to poor coping mechanisms in response to a stressful event, characterised by rapid weight gain and increased daily secretions of cortisol (Vicennati et al, 2009). Research has also suggested that lean patients suffering from PCOS have an increased concentration of plasma cortisol compared to non-sufferers and therefore may have a dysregulated HPA axis (Kialka et al, 2015; Shabir et al, 2013).

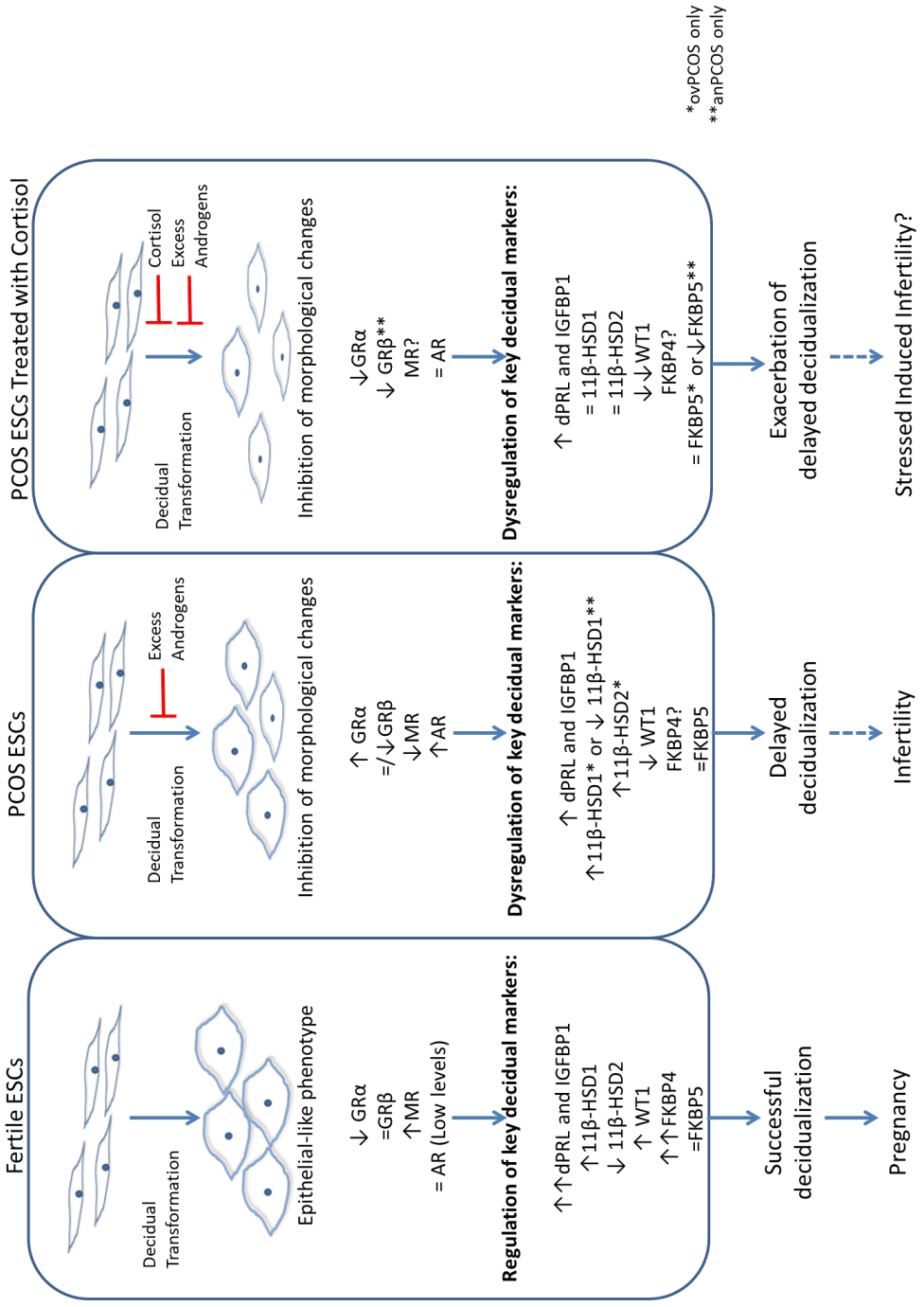

Figure 6.2 The comparison of the *in vitro* decidual response between ESCs isolated from fertile patients, PCOS patients and ESCs isolated from PCOS patients exposed to the stress hormone cortisol. ESCs isolated from fertile patients who are not exposed to the stress hormone cortisol, successful *in vitro* decidualization is able to take place due to the characteristic morphological changes of the ESCs, induction of key decidual markers and concomitant down-regulation of GRα, which can result in the successful development and maintenance of pregnancy. Whereas PCOS patients compared to fertile patients, exhibit delayed decidualization due to impaired cellular morphological changes, alterations in the GR signalling pathway and the reduction of key master regulators of the decidual response. PCOS patients exposed to the stress hormone cortisol in comparison to PCOS patients who were not exposed to the stress hormone, exhibited an exacerbated effect on the decidual response, due to inhibition of the characteristic morphological changes of the ESCs, further down-regulation of key master regulators of decidualization potentially leading to delayed decidual transformation which may result in stress induced infertility. ('↑' = indicates up-regulation, '↓' = indicates down-regulation and more arrows indicate level of up or down regulation dependent on arrow direction, '=' indicates no significant alteration in expression, '?' indicates unknown effect).

In addition to PCOS, decidualization of the ESCs has also been reported to be impaired in endometriosis patients (Su et al, 2015; Aghajanova et al, 2009), which was further confirmed by the results presented in this current study. In endometriosis patients, delayed decidualization was characterised by a slight reduction in the morphological transformation of the ESCs, although no changes in GR isoform expression or cortisol circulating levels were noted compared to fertile patients (figure 6.3 and table 6.1). This suggests that significant impairment of the decidual response is not a common feature of endometriosis patients, and may be altered dependent on endometriosis grade severity. However, the stimulation of stress exposure via the cortisol hormone resulted in the reduction of the GRβ isoform, again upsetting the fine balance of endometrial GR isoform expression which seems to be critical for decidual transformation in fertile patients. Furthermore, cortisol exposure in addition to decidualization stimulus gives rise to reduced expression levels of key decidual markers and master regulators of decidual transformation and decreased levels of 11β-HSD2, compared to endometriosis patients who were not exposed to the stress hormone, thus resulting in the exacerbation of delayed decidualization already witnessed to some extent in these patients (figure 6.3). Interestingly, altered sex steroid hormone production and signalling pathways have also been described previously in the endometrium of endometriosis patients. Similar to PCOS patients, the endometrium of endometriosis patients can also suffer from P4 resistance and aberrant PR signalling due to the chronic inflammatory environment (Patel et al, 2017). As suggested for infertile PCOS patients, dysregulation of the GR signalling pathway may be in part resulting in aberrant PR pathway signalling through potential crosstalk mechanisms resulting in modulation defects of the PR signalling pathway resulting in impaired decidual transformation being observed in endometriosis patients in response to the stress hormone cortisol.

Even though, the systemic levels of circulating cortisol remain unchanged in endometriosis patients during the secretory phase of the menstrual cycle compared to fertile patients, the investigation of cortisol exposure and its effect on infertility in endometriosis patients is still of clinical importance. This is due to the fact that detrimental effects of stress have been observed in animal models of endometriosis (Appleyard et al, 2015), and furthermore, infertility and pelvic pain caused by chronic endometriosis are considerable sources of physical and psychological adversities due to their devastating effects on reproduction, mental health and well-being, ultimately resulting in a reduced quality of life and increased stress levels (Buggio et al, 2017).

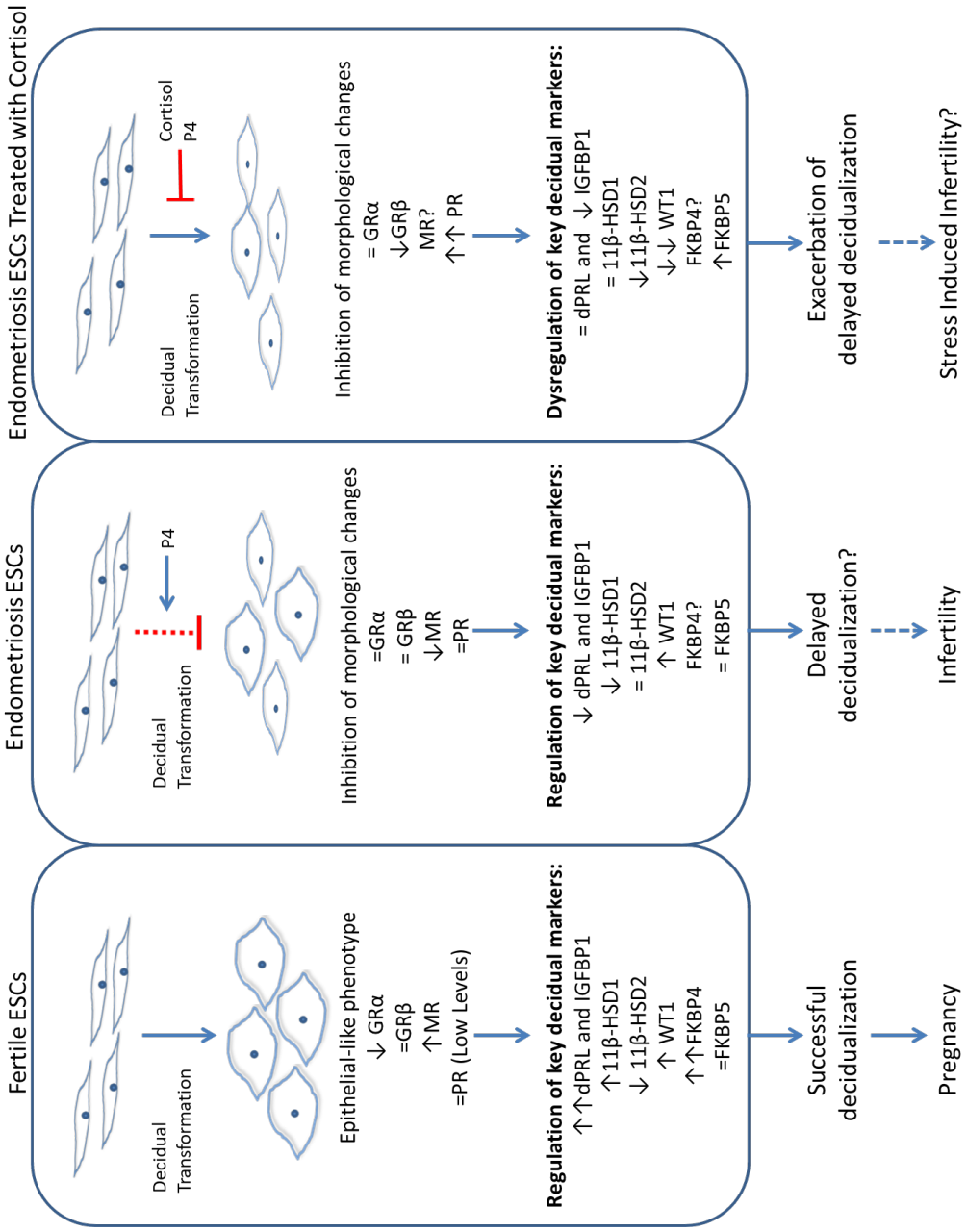

Figure 6.3 The comparison of the *in vitro* decidual response between ESCs isolated from fertile patients, endometriosis patients and ESCs isolated from endometriosis exposed to the stress hormone cortisol. ESCs isolated from fertile patients who are not exposed to the stress hormone cortisol, successful *in vitro* decidualization is able to take place due to the characteristic morphological changes of the ESCs, induction of key decidual markers and concomitant down-regulation of GRα, which can result in the successful development and maintenance of pregnancy. Compared to fertile patients, endometriosis patients exhibited potentially delayed decidualization due to impaired cellular morphological changes and alterations in key master regulators of the decidual response. Endometriosis patients further exposed to the stress hormone cortisol in comparison to endometriosis patients exhibited an exacerbated effect on the decidual response, due to inhibition of the characteristic morphological changes of the ESCs, dysregulation of the GR signalling pathway, further down-regulation of key master regulators and markers of decidualization leading to delayed decidual transformation which may result in stress induced infertility. ('↑' = indicates up-regulation, '↓' = indicates down-regulation and more arrows indicate level of up or down regulation dependent on arrow direction, '=' indicates no significant alteration in expression, '?' indicates unknown effect).

However it must be noted that the endometriosis patients in this preliminary study were not grouped by severity of disease, thus changes in cortisol levels and the GR signalling pathway between subgroups make it difficult to correlate epidemiological to molecular data obtained in this study.

In addition to endometrial infertile pathologies such as PCOS and endometriosis there is also substantial evidence to relate the psychological disposition of parents-to-be undergoing ARTs with their fertility status and thus their outcome of fertilisation techniques (Massey et al, 2014). Furthermore, prolonged stress exposure in females undergoing IVF-ET results in detrimental immunological changes resulting in an impaired stress response within the body which is associated with impaired implantation (Gallinelli et al, 2001). Demyttenaere and colleagues also indicated that women with high anticipatory state anxiety resulted in increased circulating cortisol levels and lower successful pregnancy rates in IVF treatment compared to patients with low anxiety levels (Demyttenaere et al, 1998). This is therefore suggesting that an enhanced stress response which in turn leads to alterations in the GR signalling pathway may be affecting pregnancy outcome in otherwise fertile patients. This is fully supported by the preliminary work conducted using UI patients presented in this study, which demonstrates that these patients also exhibit an aberrant expression profile of GR within the endometrial stromal compartment and increased levels of circulating cortisol during the secretory phase of the menstrual cycle, similar to that seen in PCOS patients when compared to fertile patients (table 6.1 B). Furthermore, preliminary results identify that these patients also exhibit altered levels of key decidual markers in response to the stress hormone cortisol during decidual transformation (figure 5.32). This provides evidence that the effect of stress exposure on crucial endometrial processes also needs to be investigated in detail within the UI patient cohort.

Interestingly, it is known that GCs have been used either alone or in combination during IVF treatment to improve ovarian response to stimulation and ultimately improve the outcome of IVF treatments and the number of successful pregnancies. This seems to be in contradiction to the results presented in this study, which identify that cortisol, may be an inhibitory factor of the decidual response. However, an extensive Cochrane review conducted in 2017 which aimed to determine the safety and effectiveness of the use of GCs during IVF and ICSI cycles, found that results remain unclear (Kalampokas et al, 2017). Therefore it was determined that the systemic administration of GCs may have little or no impact on the success of live birth rates. This suggests that the use of GCs during ARTs may not only have no positive effect on treatment outcome, but may be having a detrimental effect pregnancy success rates. However, it must be noted that the GCs administered during IVF and ICSI, are dexamethasone and other similar steroids and not the stress hormone cortisol, which was

investigated in this study. The differences in structure and potency of cortisol and steroids such as dexamethasone may give rise to distinct *in vivo* biological effects.

The results presented to date and the preliminary results presented in this study identify a crucial need to program fertility treatments, bearing in mind both chronic and acute stress levels, and to treat for their reduction before the commencement of fertility treatment takes place. In future, clinicians may identify stressed couples and consequently aim to reduce their stress levels by monitoring their cortisol levels not only throughout the duration of the ARTs, but also before ART has even commenced. In turn, this may diminish the number of ART cycles needed before a successful pregnancy is obtained. Furthermore, monitoring of cortisol levels may prepare the couple for an initial failure of treatment or even make the more invasive techniques unnecessary. Also, treatments and therapies to reduce stress do not provoke the ethical and religious objections raised by some clinical infertility treatments.

It is known the primary binding receptor for cortisol is GR; however, cortisol also has a high affinity for MR due to it sharing structural and functional homology with GR (Yang et al, 1997; Engeli et al, 2004). MR is known to be expressed within the human endometrium within both the stromal and glandular compartments and displays an up-regulation of endometrial expression during the secretory phase of the menstrual cycle (McDonald et al, 2006). Work presented in this study is in accordance with previous publications, which demonstrate an up-regulation of MR expression with concomitant down-regulation of GR during decidual transformation of ESCs (figure 4.8). In stark contrast, this switch of receptor dominance is not observed in infertile patients, where GR expression remains high and MR expression remains low, suggesting that in addition to the altered GR signalling observed within infertile patients, there may be an additional effect of defective MR signalling on the defective decidual response observed in the infertile patients under investigation in this study. Previous research suggests that GR and MR regulate different subsets of genes, where GR seems to repress significantly more genes than it induces during decidualization, whereas MR functions to primarily promote the expression of decidual genes (Cloke et al, 2008; Kuroda et al, 2013).Therefore, this highlights the need that further work is needed to identify GR and also MR dependent genes in differentiating ESCs isolated from fertile and infertile patients. Interestingly, it is known that progesterone activation of ESCs leads to the transcriptional regulation of distinct GR and MR gene networks (Kuroda et al, 2013). Therefore, alterations in endometrial MR expression in the infertile patient cohorts may be in part due to altered P4 and PR signalling pathways observed within the endometrium of these patients as previously discussed. Kuroda and colleagues performed a global analysis of the epigenetic status in addition to micro-array analysis to identify general pathways and genes modulated by GR and MR. However, this study conducted by Kuroda and colleagues

used the biologically inert GC; cortisone, to stimulate its conversion to the active ligand cortisol, through 11β-HSD1 activity due to its elevated expression in decidual cells as was reported in this current study (Kuroda et al, 2013; figure 6.1). Cortisone was included in this current study; however, it was deemed that the stimulation with cortisol was of increased clinical relevance and is therefore why the effect of cortisol and not cortisone exposure is the primary focus of this discussion. The use of cortisol also allows for the clarification of the effect of stress exposure on GR activation via its active ligand and the consequent effect on the decidual response within the endometrium of fertile and infertile patients.

6.2 Strengths and limitations of the study

This study successfully recruited 116 patients, which is a particular strength of the study compared to previously published literature. These patients included true fertile patients, infertile patients with known endometrial pathologies and unexplained infertile patients. The patients were able to be grouped according to pathology and also according to menstrual cycle phase, allowing for the clear identification of GR expression and the effect of the GR signalling pathway in these patient groups.

Additionally, the patients recruited to previously reported studies were undergoing hysterectomies for non-endometrial diseases which included cervical carcinomas, cervical polyps and leiomyomas and can therefore not be classed as fertile patients with no disease. However, the fertile patients recruited to this study had no pre-existing reproductive conditions which may be having a significant effect on stress levels and hence the GR signalling pathway within the body and therefore allow for the characterisation of GR expression and the GR signalling pathway in the endometrium of true fertile patients.

Despite the high number of patients recruited to this study, it was not possible to divide the endometriosis patients into grades of severity of endometriosis for the *in vitro* work, even though this was established and available, due to the patient numbers for each group becoming too low to have statistical power. Further sub-grouping of the endometriosis cohort may further elucidate how the GR signalling pathway is impacted in these patients and whether the grade and severity of endometriosis resulting in chronic pain and infertility affects this crucial signalling pathway. Additionally, small subsets of patients suffering from UI were also recruited to this study. This is mainly due to this pathology being the clinically most difficult pathology group to accurately diagnose.

It is well-characterised that circulating cortisol levels follow a diurnal circadian rhythm, which is characterised by a surge in cortisol levels immediately after awakening which

progressively decrease throughout the day (Marques et al, 2009). However a limitation of this study is that the time of day that the biopsy and serum samples were obtained was not able to be controlled. Therefore, it may be of future importance to ensure sampling procedures are standardised to allow for true comparisons between study groups. It must be noted that the majority of previously published studies investigating cortisol within the human body, do not mention if they controlled for this variable. Other external variables which may be affecting the HPA axis and consequent cortisol levels within the body include smoking, caffeine intake and food intake (Massey et al, 2014). Therefore, even though this study accounted for BMI and age, additional factors may need to be included which may improve intra- and inter- individual patient variabilities. Furthermore, this study provides compelling evidence that in addition to the previously mentioned variable external factors, it is also important to account for the phase of the menstrual cycle and whether there is any evident presence of reproductive dysfunction in females, when undertaking studies investigating cortisol and the GR signalling pathway.

An additional strength of this study is that in all experiments investigating the potential crosstalk between GR and PR utilised the P4 hormone. This is important as it is known that the synthetic derivative of progesterone: MPA which has been previously used extensively in the literature investigating GR signalling, actually introduces bias in favour of GR responses as it is a potent activator of this signalling pathway (Selman, 1996; Cloke et al, 2008).

A further limitation of the molecular work carried out in this study, was the use of Image J for the morphological analysis of the stromal cell shape. Morphological analysis was carried out by either drawing around the cells physically by hand on the Image J programme or by allowing the programme to identify cell masks. The use of Image J may introduce biased results even though a significant number of cells were examined in multiple images. Further studies would investigate the use of alternative methods to examine ESC morphology, such as In Cell analysis, identifying molecular markers which identify changes of cell shape and monitoring their expression levels via qPCR or western blot analysis.

Finally, it must also be noted that difficulties also arose when working with primary endometrial biopsies isolated from the patients recruited to this study, due to the size of the small biopsy retrieved. This led to limitations in the study in terms of the number of cells being successfully retrieved and cultured from these biopsy samples which led to some parts of the study not being able to include primary biopsy samples from patients, such as the ChIP section presented in chapter 4 (section 4.2.6.3).

6.3 Future work

Even though this body work carried out extensive investigations into the role of cortisol exposure, the GR signalling pathway and its consequent effects on endometrial decidual transformation in both fertile and infertile patients, future work is needed to identify the effect of the MR signalling pathway during decidual transformation in both fertile and infertile patients and to identify potential MR targets in differentiating ESCs.

An important consideration which has to be taken into account in future studies which involve the monitoring of cortisol levels within humans, is that there is currently not a standardised levelling system to determine whether someone has high or low levels of cortisol present (Massey et al, 2014). Depending on the study, what is determined as 'high' cortisol levels varies dramatically, hence the need for standardised system to assess reproductive function.

In addition to monitoring the serum cortisol levels of patients recruited to this study as a marker of stress, it may also be of interest to include qualitative research. Interview or questionnaire based studies will allow for the identification of patients with high perceived stress or anxiety levels and those that have an impaired quality of life. This will allow for correlation analysis between *in vivo* stress exposure and the consequent effect on GR expression and its signalling pathway in these patients.

Even though a well-established model of *in vitro* decidualization was used in this study, protein, mRNA and chromatin levels in response to decidual transformation in the presence of absence of the stress hormones were only assessed at the 48h time point. It is known that decidualization can be achieved successfully within 48h *in vitro* (Gellersen and Brosens, 2003). However, additional time points will have to be included before and after the 48h time point to ensure that the effect of the GR signalling pathway is monitored throughout the decidual response whether it is delayed or not.

Furthermore, the results presented in this study identified that *in vitro* decidualization seems to drive the expression of 11β-HSD1, which results in it becoming one of the most highly induced genes within the endometrial stromal compartment during *in vitro* decidualization. Therefore further IHC work to identify the endometrial levels of the 11β-HSD enzyme within both fertile and infertile patients is necessary. Future studies will also need to measure the enzymatic activity of the 11β-HSD enzymes in addition to monitoring their expression levels in the endometrium.

Even though this study identifies a crucial effect of the GR signalling pathway on decidual transformation in the fertile and infertile endometrium, further work is vital to identify

whether GR is crucial within endometrial decidual transformation. It would be of interest to carry out a siRNA-mediated knockdown of GR and potentially genome-wide expression profiling, which would enable us to identify how the GR signalling pathway may be related to crucial processes within the ESCs.

Extensive work was carried out to identify potential GR binding sites within the promoter regions of potential GR target genes during the decidual response using the HESC cell line (suitable model for fertile endometrium) and ChIP (chapter 4: table 4.2 – 4.4 and figure 4.25 – 4.32). However, work presented in this study only investigated the potential binding of GR to GREs located in the promoter and did not fully investigate the effect, if any, of GR binding to other TFs present within the endometrium through protein-protein tethering. In addition, it is also known that GR is able to bind to negative GREs (nGREs) (Kadmiel and Cidlowski, 2013). The investigation of this phenomenon in ESCs is of particular interest as it has been previously suggested that GR seems to repress significantly more genes than it induces during decidualization and therefore may be interacting in favour of nGREs instead of GREs (Cloke et al, 2008; Kuroda et al, 2013). Furthermore, initial experiments conducted using ChIP could also be conducted using ESCs isolated from fertile and infertile patient samples, but larger biopsies need to be isolated to ensure the high numbers of cells and chromatin needed for these experiments can be achieved.

Finally, numerous molecular techniques were employed in this study to identify and monitor the expression levels of mRNA and protein, which were time consuming and very difficult to optimise and reproduce. Future work would therefore investigate the substitution of the single analyte technologies that were used in this study for multiplex technologies, such as PCR arrays (for example the RT^2 Profiler PCR Arrays (Qiagen)) or protein arrays/chips (for example the LucidArray® (Sino Biological). The successful use of these arrays would save time, consumables and also provide results which are not only high throughput, but are also highly comparable and reproducible.

6.4 Conclusion

In conclusion, it is apparent that the GR signalling pathway plays a crucial role in reproduction in the absence of the stress response within fertile patients. However, endometrial GR expression and circulating levels of the stress hormone cortisol is altered in infertile patients diagnosed with PCOS, endometriosis and UI. The activation of GR signalling pathway due to the presence of the stress hormone cortisol is detrimental for crucial reproductive functions, including decidualization and thus implantation in both fertile and infertile patients. In addition, this study clearly demonstrates a role for the endometrial

GR and MR pathways within defective endometrial decidualization which warrants further investigation.

Therefore, in addition to the known risk factors of infertility, which include ovulatory disorders, tubal and uterine factors, failure of fertilisation and poor embryo quality, this study provides compelling evidence that an aberrant stress response may also be a significant risk factor for infertility and the outcome of ARTs. The data presented in this study support the need to reduce stress levels in infertile patients before the commencement of infertility treatments and for the introduction of stress monitoring in patients undergoing ARTs. Therefore, it is of clinical importance to not only test cortisol levels during ARTs, but to also test cortisol levels before ART has even commenced, in addition to the monitoring of other crucial steroid hormones, such as progesterone . Ideally, the development of a cortisol point of care sensor would allow for easy monitoring of stress levels before and throughout treatment durations and would allow patients to monitor their cortisol levels themselves with no need for hospital or doctor visits. In addition to monitoring cortisol levels, the use of questionnaires to identify 'perceived levels of stress' will also be of clinical relevance to identify stressed patients, who may be at risk for ART failure. Stressed patients may also benefit for stress management strategies which include holistic and psychological treatments as an approach to improve pregnancy rates in infertile couples.

Appendices

Appendix

A: Ethics - Project protocol for the use of blood in this study

IDENTIFYING BLOOD BIOMARKERS FOR GYNAECOLOGICAL PATHOLOGY

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Funding: St David's Medical Foundation grant & Departmental funds for the Reproductive Biology Group.

Abstract: Infertile women are at an increased risk of certain cancers, including cancers of the breast, ovary, and uterus. Some studies have reported anovulatory problems to be associated with increases in breast cancer, as well as with uterine cancer risk, particularly if associated with polycystic ovarian disease. Endometriosis has been found to be associated with ovarian cancers and also with breast cancer. There is a lack of blood biomarkers for the infertile patient to asses a cancer risk. The only blood investigations carried out currently are the hormonal levels but those do not mirror the causes of infertility and in particular endometrial related infertility. Furthermore there are no known biomarkers for determining predisposition of infertile women to develop gynaecological cancer. We have identified proteins (MUC1, RAGE, Galectin 3 among others) that are secreted into the bloodstream that are also expressed in the endometrium of infertile women at increased risk in developing gynaecological cancer (PCOS and endometriosis patients). Moreover breast cancer therapy Tamoxifen can activate the expression of these proteins in the endometrium. In addition a high MUC1 and Galectin 3 expression is associated with ovarian cancer progression and response to treatment. We aim to determine the level of circulating biomarkers together with

the hormonal profile in patients diagnosed with gynaecological cancer, infertility and at risk groups (tamoxifen therapy). The use of MUC1/RAGE/Galectin-3 as a panel of biomarker will positively impact on the screening, early diagnosis and intervention of infertility and gynaecological cancer, the reduction in cost associated with hospitalisation, radical surgery and IVF treatments. Moreover better diagnosis and disease screening will lead to personalised strategies and novel pathways of drug development such as development of effective antibody-based immunotherapeutic strategies.

Background:

Endometrial cancer (EC) is the most common gynaecologic malignancy and accounts for 6% of all cancers in women [1-5]. It is a highly curable disease but diagnosis is only possible through histological analysis of endometrial biopsy. Therefore screening asymptomatic women will result in unnecessary additional biopsies. Currently, there is no screening test that is accurate and reliable enough to detect EC in the general population. Numerous studies have demonstrated that infertile women are at an increased risk of developing gynaecological cancers [6-7]. Anovulatory problems particularly if associated with polycystic ovarian disease (PCOS) are associated with breast and endometrial cancer [7-9]. An increased incidence of EC has been found in association with prolonged, unopposed estrogen exposure and with tamoxifen treatment of breast cancer (BC), due to the estrogenic effect of tamoxifen on the endometrium [10-11]. Around 48,000 British women develop breast cancer each year, including 35,000 after the menopause, and Tamoxifen has been routinely used for 20 years to cut recurrence rates [12-13]. Recent data suggests that the optimum adjuvant therapy for postmenopausal women with BC is tamoxifen followed by aromatase inhibitor, rather than aromatase inhibitor throughout [14]. A panel of biomarkers that helps in early diagnosis would therefore be useful especially for high risk groups, *e.g.* women who are on tamoxifen treatment and PCOS patients.

Ovarian cancer (OC) is the 5th most common cancer in women (6,500 new cases/year, UK) after breast, bowel, lung and womb cancer [5]. The risk of OC is considered to be increased in patients with a family history of OC, BC patients, infertile women and those who had fertility treatment, HRT, obesity, endometriosis, smoking and diet factors [15]. Despite the development of new treatments and therapies designed to improve the five-year survival rate, ovarian cancer still remains the deadliest cancer of the female reproductive tract [16]. Unfortunately, most cases are diagnosed in the late stages of the disease, when the five-year survival rates fall below 20%. The lack of precise early warning signs is one of the factors that further contribute to the fact that only 25% of ovarian tumours are identified at stage 1 [17]. As most cases present in late stages of the disease, few opportunities are present for

treatment and to ultimately improve survival. Although a serum test for CA125 is available for monitoring the disease, this test lacks the specificity and sensitivity for early detection [16]. Clinical and epidemiological studies have reported an association between Endometriosis and ovarian and breast cancers [7, 18-21]. There is lack of blood biomarkers for these infertile patients.

Altered glycosylation on the surfaces or secreted proteins of tumour cells is a common feature of breast and gynaecological cancers (EC and OC) [17, 22-23]. Cancer-associated glycans are often found on a class of proteins called mucins [24, 25]. Epithelial mucin 1 (MUC1) is transmembrane glycoprotein of the mucin family normally expressed on the apical side of healthy epithelial cells. In malignant cells, with transformation and loss of polarity MUC1 is aberrantly overexpressed and underglycosylated on the entire cell surface of 90% of BC cells and other cancer types [26]. The proteolytic shedding of the extracellular domain of MUC1 into the bloodstream rends the immunodominant epitope MUC1 (CA15-30, also known as a tumour marker antigen. An increase in the serum CA15-3 shed ectodomain is associated with progression of carcinoma in patients diagnosed with BC [27]. In BC cells MUC1 overexpression correlates with Estrogen receptor (ER) expression and MUC1 associates with ER complexes on estrogen-responsive promoters, increases recruitment of coactivators and antagonises the inhibitory effects of the antiestrogen tamoxifen [28]. Therefore the net effect of tamoxifen will potentially be influenced by the expression levels of MUC1. A two to three fold increase in the risk of developing EC is associated with tamoxifen treatment in BC patients [11]. We have preliminary data that shows MUC1 is overexpressed in endometrial cells exposed to tamoxifen, effect not observed in breast cells. Additionally we have unpublished data that shows upregulation of MUC1 endometrial expression by advanced glycation endproducts (AGEs), metabolites that are found at high levels in the bloodstream of PCOS patients. Finally, we have recently published that a different glycosylation pattern of endometrial MUC1 subunits and epitopes is observed between fertile and infertile women diagnosed with endometriosis and PCOS [29].

Endometriosis is defined by the presence of tissue implants resembling endometrial glands outside of the uterus, at ectopic sites, frequently on the ovarian surface. The ectopic lesions are often invasive, resistant to therapy, and may predispose to endometrioid and clear cell ovarian tumours [30]. MUC1, which is normally present on eutopic human endometrial glands, is also present in ectopic lesions of endometriosis. Furthermore, changes in MUC1 expression in endometriosis could promote adaptive anti-MUC1 immunity that might play a role in the malignant progression [31]. Several studies have highlighted the link between the appearance of OC and BC with a previous clinical history of endometriosis [7, 18-21]. We

have published that MUC1 expression is altered in eutopic endometrium of endometriosis patients [29].

It will be interesting then to determine MUC1 (CA15-3) bloodstream levels in patients at risk of developing gynaecological cancer (infertile and BC on tamoxifen) and compare to the levels of patients suffering with EC or OC.

We will extend our studies to the analysis of other putative biomarkers recently identified by us in endometrium. We have identified the increased expression of inflammatory mediators such as the receptor for advanced glycation end products (RAGE) and Galectin 3 (Gal-3) in the endometrium of PCOS patients and in endometrial cells exposed to tamoxifen. RAGE and its ligands are intimately involved in the pathobiology of a wide range of diseases that share common features, such as enhanced oxidative stress, immune/inflammatory responses, and altered cell functions [32, 33]. Soluble forms of RAGE (sRAGE), including the splice variant endogenous secretory (es)RAGE, have been found circulating in plasma and tissues. Experimental data suggest that these isoforms may neutralize the ligand mediated damage by acting as a decoy. Increased expression of RAGE was demonstrated in different tumours including breast cancer and may increase with the progression of the tumour [32, 34]. Circulating sRAGE levels may inversely reflect RAGE activity, thus providing a useful biomarker of RAGE mediated pathogenesis. Increasing evidence indicates that RAGE has also an important role in cancer. In cancer cells ligand activated RAGE can directly stimulate proliferation, invasion, angiogenesis, chemoresistance, and metastasis [32]. The blockade of the ligand-RAGE engagement in vitro suppresses tumour proliferation and invasion. Consistent with these hypotheses, Tesarova´ et al. [35] find decreased sRAGE levels in patients with breast cancer compared to healthy controls, with higher levels in patients with low-grade cancer and positive estrogen receptors. These data suggest that sRAGE may be a molecular link underlying the epidemiological association between pathologies where RAGE is active such as diabetes and cancer. A common feature between diabetic and PCOS patients is high circulating levels of AGEs. An increased expression of RAGE receptors by AGE or glucose levels has been described in several tissues including the endometrium (our unpublished data). Interestingly Gal-3 expression and secretion has been demonstrated to be induced by RAGE ligands (AGE) and high glucose milieu [36]. Recognition and binding of AGE products is one of the many functions of Gal-3, just like sRAGE, that provides cell protection against the AGE/RAGE induced-cell injury [37]. New published evidence suggests that Gal-3 is overexpressed in various forms of endometriosis, highlighting the potential of this protein to emerge as a novel biomarker for infertile pathologies [38]. Increased concentrations of free circulating Gal-3 are commonly seen in the blood circulation of patients with many types of cancers. Gal-3 concentration was also significantly

higher in the serum of patients with breast [39], lung [40], head and neck [41] cancers and melanoma [42]. Furthermore, patients with metastatic disease have higher concentrations of circulating Gal-3 than those with localized tumours [39]. Interestingly, the metastatic effects of galectin-3 are thought to be related to the binding of galectin-3 to the Thomson-Friedenreich carbohydrate (galactose β1, 3N acetylgalactosamine-, TF) antigen expressed by MUC1 [43]. We have preliminary data showing that Gal-3 expression is increased in endometrial cells exposed to tamoxifen and several studies have highlighted the elevated levels of circulating Gal-3 in BC patients. It will be important to determine whether levels of Gal-3 can be associated to the appearance of endometrial pathologies or the progression from endometrial related infertility to gynaecological cancer. Targeting Gal-3 actions in the circulation may hold significant promise for future development of novel therapeutic agents to prevent metastasis and reduce cancer-associated fatality.

MUC1, RAGE and Gal-3 are regulated by ER and its ligands. We have published that infertile endometrium expresses very high levels of ER (29). During the fertile years endometrial ER expression is upregulated by estrogens whereas progesterone downregulates its expression. After the menopause when the risk of developing endometrial cancer increases, expression of ER and its targets may possibly be regulated by external sources of estrogens and progesterone such as those given during HRT treatment. In fact, a high risk of developing breast cancer and ovarian cancer is associated with the prolonged use of combined estrogen and progestin (progesterone synthetic derivates) HRT treatment [44]. The risk is associated mainly to the use of progestins instead of natural progesterone, which has low solubility and poor bioavailability compared to its synthetic derivatives. Progestins are also use for attenuating heavy or prolonged menstrual bleeding, as a contraceptive, and for the treatment of endometriosis. In theory all these patients groups may also be at risk of developing gynaecological cancer. Taking these factors into account we will also determine the levels of circulating hormones in patients at risk of developing gynaecological pathologies.

The use of MUC1/RAGE/Gal-3 as biomarker will impact in the screening, early diagnosis and intervention of infertility and cancer, the reduction in cost associated with hospitalisation, radical surgery and IVF treatments. Moreover better diagnosis and disease screening will lead to personalised strategies and novel pathways of drug development such as developing of effective antibody-based immunotherapeutic strategies.

Statement of the problem:

EC is the fourth most common cancer in women (20.7/100,000), and the most common gynaecological cancer [1-5]. Ovarian cancer is the 5th most common cancer in women, and

remains the deadliest cancer of the female reproductive tract with a five-year survival rate [26]. Current test design to monitor ovarian cancer (CA125 serum levels) lacks the specificity and sensitivity for early detection while there is no test available to monitor EC. An increased incidence of EC has been found in association with tamoxifen treatment of BC. More than 48,000 women in UK have BC diagnosed every year and up to 80% will be offered treatment with Tamoxifen. Therefore there is a need for identification and development of biomarkers to improve diagnosis and prognosis. Infertile women are at an increased risk of certain cancers including ovary and uterus. Anovulatory infertility such as PCOS has been identified to be associated with risk of developing endometrial cancer whereas endometriosis has been linked to ovarian cancer. About 1/5 women in the UK has polycystic ovaries, and ~1/10 has PCOS to some degree. Endometriosis affects ~2 million women in the UK. There is no known cure for endometriosis. It is a chronic debilitating condition that causes pain and fertility problems. We aim to characterize the role of pro-inflammatory mediators in infertility and in progression from infertility-associated pathologies to gynaecological cancers.

AIMS:

We aim to determine the levels of circulating biomarkers together with the hormonal profile in patients at risk of developing gynaecological cancers and to monitor appearance/progression of disease in cancer patients. We have working protocols and ethical approval for ex vivo culturing and processing of patient samples and data analysis (study groups: fertile, infertile endometriosis, PCOS, EC, postmenopausal bleeding, and BC patients taken tamoxifen).

Research question: We want to investigate the role of MUC1/RAGE/Gal-3 as a determinant of cancer progression and infertility associated with endometriosis and PCOS; both risk groups for development of ovarian or endometrial pathology respectively.

Primary/Secondary Outcome Measurements:

Primary Outcome

1. Our research will study the blood levels of MUC1, RAGE, Galectin-3 and other biomarkers in infertile patients, patients with endometrial pathology and controls, assess the sensitivity and specificity of these markers in predicting infertility status, endometrial or ovarian pathology and disease progression.

Secondary Outcomes

1. Establish any correlation between expression of biomarkers in blood and endometrial tissue. Determine the value of the blood biomarker examination along with (or instead of) the endometrial biopsy as predictive for further pathology.

2. The levels of MUC1, RAGE and Galectin-3 and other biomarkers will be assessed in combination with the levels of circulating hormones: estrogen, progesterone and gonadotropins which are routinely measured in clinical practice to determine type of infertility, to determine if the use of these additional biomarkers can improve diagnostics.

Methodology:

Patients presenting to the Gynaecology, Gynaecology –Oncology or Infertility clinics will be invited to participate in the study and donate a blood sample which will be subsequently processed in our laboratory for identification of the above mentioned biomarkers and hormones.

1. Information leaflets will be send to patients a week prior to attendance to clinics. Suitable patients will be identified by the clinicians from the Gp referral letters. The patients will then be approached on attendance to clinics. A member of the research team will explain the study to the woman and obtain a written consent. Also the research team member will ask the patient some basic questions about the reason for attendance to clinic, their medical history and medication.

2. For the patients that are booked for a procedure or have bloods taken as part of their investigations the blood sample will be taken at that time so no extra needles or injections are required.

3. For the patients that will not have a blood sample taken as part of their further care, if they agree to be part of the study a sample will be taken in clinic.

4. 15-20 ml of blood will be taken from the patients.

5. Laboratory based analysis [ELISA for serum levels of biomarkers]. Blood will be transported to our laboratory in the Institute of Life Science at Swansea University for the research project.

Tools for data collection:

Data collection will be performed using a proforma (see attached Appendix) containing all patients' demographic and clinical information.

Data Entry: All reported data will be entered via a secure (password protected) anonymised web-based study database and accessed only by the members of the research team.

Inclusion/Exclusion criteria:

For the study, **inclusion** criteria are broad so consecutive patients attending the Gynaecology Department will be invited to participate in the study. Based on the diagnosed pathology or reason for attendance (e.g. request for sterilisation), they will be allocated to the infertility, endometrial pathology or control groups.

The following enrolment **exclusion** criteria apply:

- Patient is pregnant or breastfeeding.
- Known non gynaecological malignancy
- Documented evidence of ongoing infection.
- Women declining to consent.
- Women unable to give informed consent.
- Women for who English is not their first language and who are unable to fully understand the verbal and written information about the study.
- Adolescents under the age of 18.

Ethical Considerations:

Informed Consent

Prior to any data collection under this protocol, a written informed consent must be signed by the patient, in accordance with local practice and regulations. Information about the study will be explained to the patient. A copy of the informed consent form, signed and dated by the patient, must be given to the patient. Confirmation of a patient's informed consent must also be documented in the patient's medical records prior to any data collection under this protocol. This is a non-interventional study; therefore, the risks for subjects linked to their

participation in the study are limited to a breach of confidentiality with regard to personal identifiers or health information.

Privacy and Confidentiality

In order to ensure patient confidentiality, patients will be assigned a unique identifying number. Upon enrolment, patients will be required to provide their name and address. The patients' name and contact information will not be entered into the study clinical database. The database containing the clinical details and experimental results will be password protected and accessed only by the members of the research team. The chief investigator (Dr. Margarit) will store the patients' name and identification information separately from other study information in locked secured storage area (in the investigator's office). The unique study number will be the only identifier visible to all the other members of the research team.

Ethics Committee

Prior to the collection of any study related data, approval of the protocol, informed consent and all patient enrolment materials will be obtained from the required Ethics Committee.

Protocol Modifications

N/A

Benefits of the Study:

We will be able to compare the circulating levels of the proposed putative biomarkers in cancer patients and infertile patients at risk of developing gynaecological cancers and identify patterns of expression common to both pathologies.

A high proportion of women with PCOS and endometriosis will be referred for IVF treatment, despite a poor prognosis. IVF is expensive; implementing the NICE recommendation of one cycle of treatment for every couple is ~£1.4M/annum in Wales. Elucidation of biomarkers levels in fertile vs. infertile patients may permit clinicians to identify those who have the best chance of IVF success, allowing limited resources to be focussed on these women thus offering a significant advance in service organisation and delivery for fertility treatment.

The ability to develop patient tailored investigations, diagnostic tools and treatments guided by clinical and molecular evidence will contribute to more effective management consistent with the Wanless Report aims [45].

Prevention and early intervention

Characterization of MUC1/RAGE/Gal-3 role as biomarkers for gynaecological cancer will enable early intervention to prevent disease progression. Interventions are available for EC to reverse pre-cancerous changes in the endometrium, the development of a test to define high-risk groups would enable earlier presentation when surgery alone is likely to be curative, thus avoiding the long-term side effects of adjuvant radiotherapy for more women. This research will contribute to reducing cancer incidence, minimising avoidable death and pain; WG priorities for healthcare in the next 5 years [46, 47].

Recruitment:

Recruitment of patients into the study will be performed by clinicians (members of the research team) based at Singleton, Neath Port-Talbot and Princess of Wales Bridgend Hospital.

Sample size

There is relatively little prior research in this area on which to base a power calculation. However, our previous study on these markers in broadly in agreement with similar work in related areas.

Previous research on MUC1 levels in blood samples [48] shows raised levels in cancer patients when compared to controls (37% and 11% positive tests respectively). When working with a 5% level of significance sample group sizes of 47 would be necessary to ensure deliver 80% power. Our research into MUC1 levels in biopsies from infertile patients [29] indicated large variation in mean (standard deviation) level of expression between controls (13.2(2.1)) and each of the infertile subgroups (14.3(1.8), 11.5(4.3), 12.1(2.4)). For 5% significance and 80% power, a sample group size of 89 would distinguish between every one of these subgroups individually and the healthy controls.

Although the cancer study focussed on Breast rather than gynaecological cancer and the second study focussed on infertility, we feel that they indicate the high level of variation in MUC1 levels (in biopsy and blood) between healthy and compromised subjects. We also believe that the other indicators RAGE and Galectin-3 will show similar strong links to patient health. Caution dictates that we assume all unknown factors will act against but we would argue that a sample size of between **150 and 200 patients** should ensure significant variation for both blood and biopsy measures.

As for the correlation between the biomarkers levels in the biopsies and in blood, there is no previous research on which to base our expectations. As such this part of the research is entirely exploratory and no meaningful sample size calculation can be performed. It is

however expected that if the levels in both biopsy and blood vary significantly among the groups of interest then any correlation that exists between them should be measurable.

Timescale

We anticipate that the target number of patients for this study [200 patients] will be recruited over a period of 3 years and another 6-12 months will be needed for data analysis and dissemination of results in scientific journals. Therefore we propose a timescale of 4 years for the completion of this study once ethical approval is granted.

Analysis of the results:

Statistical analyses will be descriptive and will aim to characterize the study population, disease management patterns, as well as analysing the biomarkers' levels and its relationship to the pathology status.

Continuous variables (age, BMI) will be summarized in terms of means, standard deviations, medians, minima, and maxima for continuous variables. Categorical variables will be reported as frequencies and percentages. Occurrences of events (e.g., performance of surgery, onset of endometrial thickening) will be reported as rates and supplemented with two-sided 95% confidence intervals to reflect the uncertainty in the estimate. These results will be accompanied by p-values to facilitate the interpretation of the statistical significance of the findings.

Conventional evaluation methods [sensitivity and specificity] and general linear models will be used to predict the value of the biomarkers in identifying infertility and gynaecological cancer. We would study the correlation between levels of biomarkers in biopsy and blood for the same patient using Spearman's Rank Order Coefficient.

Possible problem areas conduction the study:

One potential problem might be encountered when recruiting patients for which is a blood sample is not required as part of their further care, in which case taking this sample for research purposes will be an extra intervention.

Dissemination of research findings:

The dissemination of results will be through presentations to relevant scientific meetings and publications to peer review journals.

Representative consumers' involvement

There is no involvement of representative consumers in the design process of this research project. The patients and their health providers will not be informed of the results of this research as this is not a diagnostic study. The patients might be under continuous treatment and the results of this research will not be meant to influence the already planned treatment. If they wish, the patients can be sent copies of the research papers and presentations abstracts that might emerge from this study.

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B: Ethics - Project protocol for the use of endometrial biopsies in this study

Establishment of primary cell cultures using biopsy samples of human endometrium as model to investigate differentiation and preparation for implantation

Project protocol

Aims

To identify and characterise molecular biomarkers and signalling networks which contribute to the differentiation and successful implantation. To investigate the markers and pathways involved in endometrial infertility in a range of conditions including polycystic ovary syndrome, endometriosis and unexplained infertility.

Recruitment

Patients will be recruited from gynaecology outpatient clinics, and pre-admission clinics. Formal written consent will be obtained and documented in the patient's notes. The following groups of patients will be approached and given both verbal and written information:

- Group 1. Fertile women [Gynaecology clinic or theatre e.g. sample from hysterectomy specimens for benign disease such as prolapse]
- Group 2. Patients with PCOS [Gynaecology clinic]
- Group 3. Patients with endometriosis [Gynaecology clinic]
- Group 4. Patients diagnosed with unexplained infertility[Gynaecology clinic]

Exclusion criteria

Women declining to consent

Women unable to give informed consent

Women for whom English is not their first language and who are unable to fully understand the verbal and written information about the study.

Adolescents under the age of 18.

Handling of samples

The tissue samples will be anonymised and the anonymisation information kept by Dr Margarit. The samples will be subject to a number of contemporary molecular biology techniques including, but not limited to:

1. Tissue Microarrays to detect the transcription factors which regulate the molecular pathways of the cells.

2. Chromatin Immuno-Precipitaion to assess the function and abundance of these transcription factors.

3. Tissue cell culture to assess the interplay between the regulatory pathways.

4. Bioinformatic meta-analysis to identify gene sets common to each pathway.

In the event of results which are found to be clinically relevant to an individual patient, this information will be passed onto the patient's consultant or multidisciplinary team.

C: Ethics – Information sheet for research participants and consent form – Blood samples

Patient's copy of the information sheet

IDENTIFYING BLOOD BIOMARKERS FOR GYNAECOLOGICAL PATHOLOGY

We are inviting you to help us with this research project. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like further information. Take time to decide whether or not you wish to take part

What is the purpose of the study?

The aim of this study is to find out if a range of substances in the blood, which we call a panel of biomarkers can have a role in screening, early diagnosis and treatment for both infertility and gynaecological cancer,

To do this we would like to take a small amount of blood equivalent to two tablespoons and process it in the laboratory. Blood tests have been used before in other studies but none have looked at the factors and processes we wish to study.

Why have I been chosen?

Women who come to the Obstetrics, Gynaecology, Gynaecology–Oncology or Infertility clinics have health problems which make their blood useful to us to test. If you need to have a blood sample as part of your planned care, the sample for the research can be taken at the same time. This leaflet has been sent to you ahead of your attendance to clinic to give you time to consider whether or not you would like to participate in this research study.

Do I have to take part?

Nobody has to take part in this research study, it is up to you to decide whether or not you would be happy to help. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. After signing the form, you are still free to change your mind and withdraw from the study at any time without giving a reason. A decision to withdraw at any time or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

When you come to your gynaecology clinic, a member of the research team will explain the study to you, go through the consent form and ask you some basic questions about the reason you needed to come to clinic, your medical history and medication. This information will be written on a form that will carry a unique code (same code that will be given to the blood sample). Members of the research team will have access to this information but will not have access to any of your identifying personal details as name, address, post code.

If you need to have blood taken as part of your normal care, the research blood sample will be taken at the same time so you have no extra needles used.

If you do not need a blood sample taken as part of your normal care, if you agree to be part of the study, a sample will be taken either straightaway in clinic or at one of your normal follow up clinic appointments.
After the samples have been anonymised and analysed we will need to get information regarding your diagnosis from the medical team that is looking after you. The research team will need to have access to this information. However, this process will not affect the patients' confidentiality.

What do I have to do?

In total, you would answer some basic questions about your health, sign a consent form and give one blood sample.

What tests will be performed?
We will use a laboratory technique to determine if a certain substance is present in the blood sample. Genetic tests might be done but these will only focus on providing information about the disease and not about individuals. It will not affect you or your family. Much research compares genetic material from people with known disease and those without disease. This comparison helps to reveal genetic differences that may be used to develop new tests.

Finding out the significance of the tests is the purpose of out study, so your own results have no meaning for your healthcare and will not be given to you or your doctor.

What are the possible disadvantages or risks of taking part?

If you need a blood test as part of your normal care, there are no additional risks.

If you don't need a blood test as part of your normal care, then giving a sample is an added task for you. Sometimes it can be difficult to find a vein to draw blood, some people feel faint and some have a bruise afterwards. However, there are no serious dangers.

What if something goes wrong?

If you were to have any problem as a consequence of your participation in this study, you would be compensated through the Hospital "No Fault" Compensation Scheme. Your right at law to claim compensation for injury where you can prove negligence is not affected.

If you wish to complain or have any concerns about any aspects of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanism will be available to you.

Will my participation in this project be confidential?

All information that is collected about you during the course of the research will be kept strictly confidential. Any information that leaves the hospital will not have your personal details attached, so you could not be recognised from it. Only members of the research team will have access to personal or clinical data.

What will happen to the results of the research study?

The results will be published in peer reviewed journals and presented at conferences. You can obtain a copy of the published results, if you wish, from the office of Prof RS Conlan at Swansea Clinical School. No person taking part will be named in any report or publication.

Who is organising and funding the research?

Prof Conlan, Head of Reproductive Biology, School of Medicine, Swansea University, has organised the laboratory aspect of this study and is supported by funding from the Welsh Office of Research and Development.

Who has reviewed this study?

The South West Wales Research Ethics Committee has reviewed the study.

Please feel free to ask us any questions. Dr. Margarit is co-ordinating this research project. If you require any other information please contact her secretary on telephone number 01656 752970. If you wish to discuss this study with a Consultant Gynaecologist that is not involved in this research please contact Miss. Hilborne Consultant Gynaecologist in Princess of Wales Hospital. You can contact her on the telephone number 01656 752970.

Study ID Number:

Consent form

Title of project:

IDENTIFYING BLOOD BIOMARKERS FOR GYNAECOLOGICAL PATHOLOGY
(version 4.1 April 2016)
The participant should complete the whole of this sheet herself.

(Please INITIAL each statement if it applies to you)

I have read the Information Sheet for Patients (version 4.1, 26 April 2016). ☐

I have been given the opportunity to ask questions and discuss this study. ☐

I have received satisfactory answers to all my questions. ☐

I have received enough information about the study. ☐

The study has been explained to me by:
Prof/Dr/Mr/Mrs/Ms_____

I understand that I am free to withdraw from the study at any time, without having to give a reason for withdrawing and without affecting my future medical care. ☐

I agree to take part in this study. ☐

I understand, the study using the sample I give may include genetic research aimed at understanding the genetic influences on gynaecological pathology but the results of these investigations are unlikely to have any implications for me personally. ☐

Signed.....Date.....

(NAME IN BLOCK CAPITALS).....……..

Investigator's signature.....…Date: .....

(NAME IN BLOCK CAPITALS).……...

D: Ethics – Information sheet for research participants and consent form – Endometrial biopsy samples

Information Sheet for Research Participants

Patient's copy of the information sheet

The establishment of endometrial cell culture study

We are inviting you to participate in the above research project. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like further information. Take time to decide whether or not you wish to take part

What is the purpose of the study?

The lining of the womb (endometrium) undergoes changes throughout the monthly cycle and one of them is preparation for implantation of a fertilised egg. Abnormalities in this process may cause failed implantation and thus lead to infertility.
Little is known about the factors and processes that control these changes in the endometrium. The aims of this study are to

a) Find out what are the factors that regulate the preparation of the endometrium for implantation and,
b) Investigate how they work

To do this we will take tiny fragments of the endometrium and grow them in the laboratory in order to investigate these factors. This approach of endometrial cell culture has been used before in other studies but none have looked at the factors and process we wish to study.

Why have I been chosen?

We need to take tiny fragments of tissue at any phase of the menstrual cycle. Your gynaecologist has advised you to have an operation, either a hysteroscopy alone or as part of another operation, or a laparoscopic clip sterilisation. Taking a tiny fragment in addition to your planned operation would be a simple procedure without causing you any harm.

Do I have to take part?

Taking part in this research study is entirely voluntary and is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to change your mind and withdraw from the study at any time without giving a reason. A decision to withdraw at any time or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

You are a patient undergoing a hysteroscopy either alone or as part of another operation or a laparoscopic clip sterilisation.

During a hysteroscopy the cervix is gently stretched to allow the camera to pass and then a biopsy of the endometrium is taken using an instrument called a currette.
During a laparoscopy a fine instrument is placed through the cervix in order to move the uterus.
If you agree to participate to this study, an endometrial biopsy will be taken, by a standard gynaecological curette at the end of your scheduled procedure.
Only a small amount of tissue will be necessary, less than the size a few 'nail clippings'. Each patient who agrees to participate in the study will have only one sample taken.

What do I have to do?

You have been given information about your planned operation and having the biopsy will not add any further restrictions.

What tests will be performed?

The endometrial biopsies will be processed to separate the two structural different types of cells. These will be then grown for 1-3 weeks. They will undergo different treatments to mimic physiological and pathological conditions. At the end of their treatment the cells will be analysed for gene expression. We wish categorically to state that this will not involve DNA testing.

What are the possible disadvantages or risks of taking part?
If you are in the group of patients who require a biopsy as part of your investigation, there are no additional risks beyond those associated with your scheduled operation.
If you will have an endometrial biopsy for research purposes only, the cervix will already have been entered or dilated. The possibility of uterine perforation that might occur under these conditions is likely to be <1:1000.
There is a minimal risk of pelvic infection after any endometrial biopsy and if you have any offensive vaginal discharge/heavy vaginal bleeding/ abdominal pain you should contact Ward 20 on 01792205660 ext 5721.

What if something goes wrong?

In the event of you suffering any adverse effects as a consequence of your participation in this study, you will be compensated through the Hospital " No Fault" Compensation Scheme. Your right at law to claim compensation for injury where you can prove negligence is not affected.
If you wish to complain or have any concerns about any aspects of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanism will be available to you.

Will my participation in this project be confidential?

All information that is collected about you during the course of the research will be kept strictly confidential. Any information about you that leaves the hospital will not have your personal details attached, so you cannot be recognised from it. The data will be handled only by those directly involved in the study. We will not inform the GP about the study unless you wish.

What will happen to the results of the research study?

The results will be published in peer reviewed fertility journals and presented at conferences. You can obtain a copy of the published results, if you wish, from the office of Dr. Steven Conlan at Swansea College of Medicine. You will not be identified in any report or publication.

Who is organising and funding the research?

Prof. Steven Conlan, Head of Reproductive Biology Group, College of Medicine, Swansea University, has organised the laboratory aspect of this study and is supported by Medical School funds.

Who has reviewed this study?

The Bridgend Neath Port Talbot and Swansea Local Research Ethics Committee has reviewed the study.

Please feel free to ask us any questions. Dr. Margarit is co-ordinating this research project. If you require any other information please contact her on Tel. No. 01656752308.

Title of project:

Establishment of Endometrial Cell Cultures

The participant should complete the whole of this sheet herself.

(please tick each statement if it applies to you)

I have read the Information Sheet for Patients and Healthy Volunteers. ☐

I have been given the opportunity to ask questions and discuss this study. ☐

I have received satisfactory answers to all my questions. ☐

I have received enough information about the study. ☐

The study has been explained to me by:
Prof/Dr/Mr/Mrs/Ms_____

I understand that I am free to withdraw from the study at any time, without having to give a reason for withdrawing and without affecting my future medical care. ☐

I agree to take part in this study. ☐

Signed.....Date.....

(NAME IN BLOCK CAPITALS).....……..

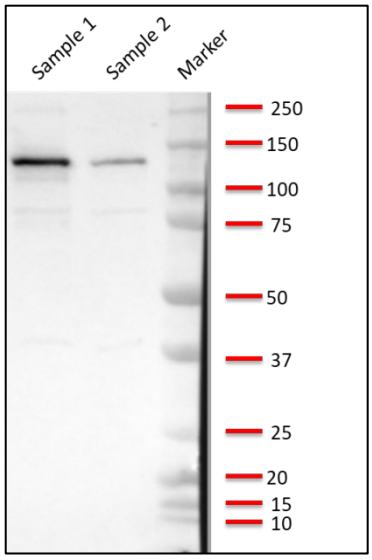
Investigator's signature.....…Date: .....

(NAME IN BLOCK CAPITALS)……...

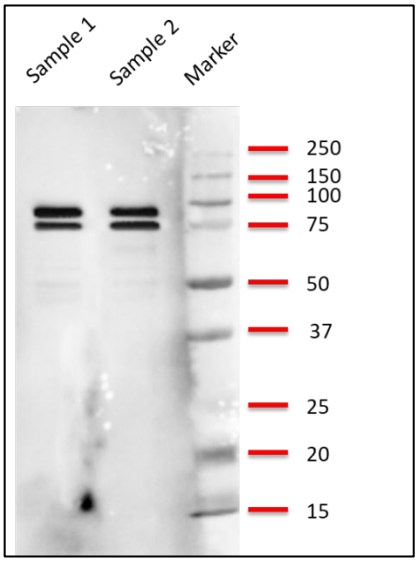
E: Images of Immunoblots of all antibodies used in this study, alongside the molecular weight marker (Precision Plus Dual Colour Standard, Bio Rad)

Full size images of the western blots were carried out to validate the quality of all antibodies used and to compare expected molecular weight compared to the actual observed molecular weight of the targets. A: Vinculin, B: GR, C: AR, D: PR, E: ER (more than one band observed which indicates the presence of alternative ERα isoforms i.e. ERα46 and ERα36), F: 11β-HSD1, G: 11β-HSD2, H: PRL, I: WT1 and J: FKBP51.

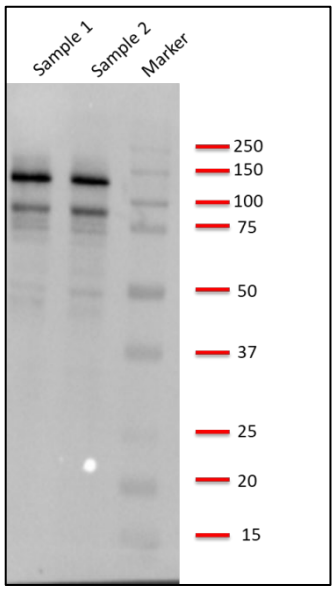
A: Vinculin (124kDa)



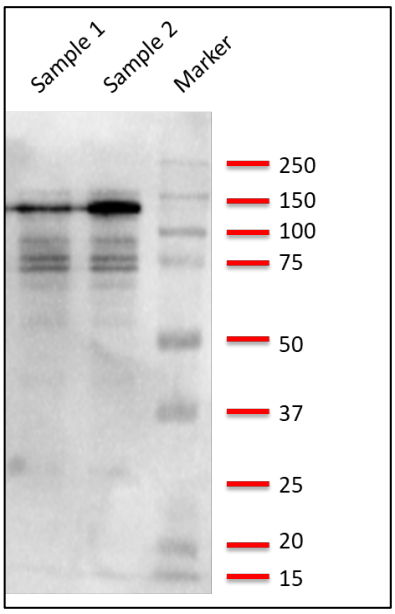
B: GR (95/90 kDa)



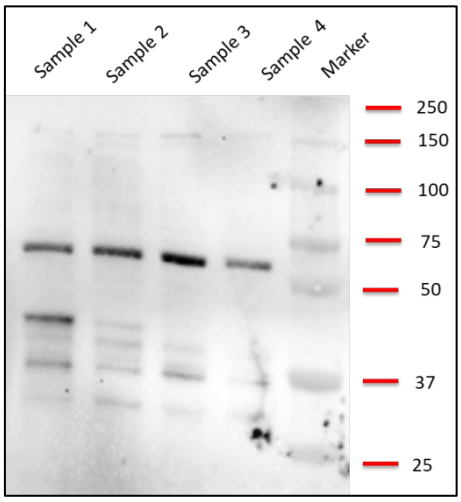
C: AR (110/75 kDa)



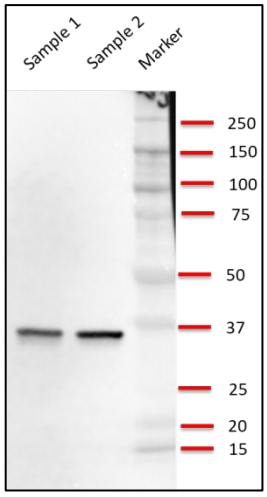
D: PR (116/81 kDa)



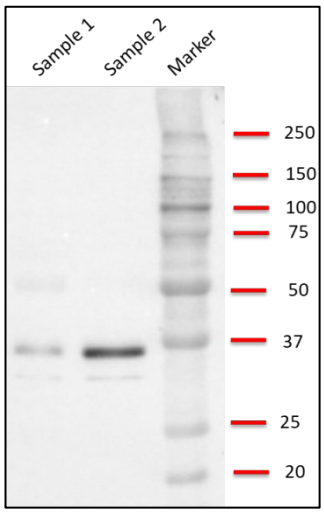
E: ER (66kDa)



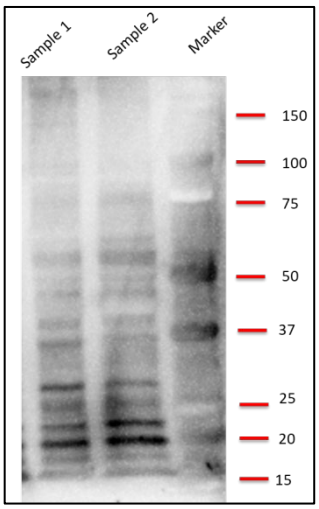
F: 11β-HSD1 (32kDa)



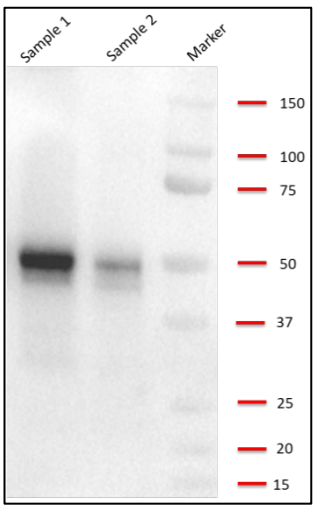
G: 11β-HSD2 (40kDa)



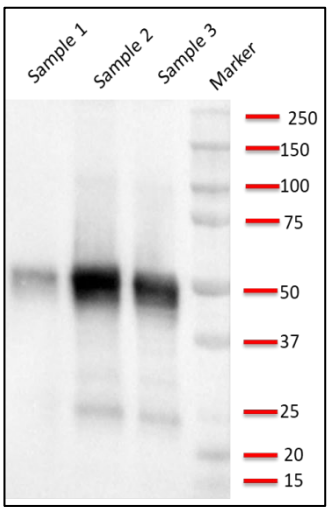
H: PRL (27kDa)



I: WT1 (52kDa)



J: FKBP51 (51kDa)



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