Hydrocortisone and TGF-ß1 regulate the glucocorticoid receptor and the serotonin transporter in SWAN-71 and JEG-3 cells

Hydrocortisone and TGF-β1 regulate the glucocorticoid receptor and the serotonin transporter in SWAN-71 and JEG-3 cells

Dylan Robertson

BBiomedSc

Griffith University Health Group

School of Medical Science

Griffith University

Principal Research Supervisor: A Prof Roselyn Rose'Meyer

Associate Research Supervisor: A Prof S. Niru Nirthanan

Submitted in fulfilment of the requirements of the degree of

Master of Medical Research

27th February 2019

Acknowledgements

I would like to thank my supervisor, Associate Professor Roselyn Rose'Meyer, for the opportunity of taking on this project and conducting independent research. Your expertise, guidance, and support throughout the year has been invaluable and has allowed me to grow as a scientist, student, and person. Thank you.

I also wish to express my gratitude to Associate Professor S. Niru Nirthanan, who has always made himself available and provided guidance during both the research proposal and final thesis write-up periods.

I must also thank Dr. Jelena Vider for the technical expertise and advice throughout the experimental process. I feel very fortunate for having the opportunity to learn from and work with you.

A special thank you to my colleague, Mr. Samuel Hall, for the constant support throughout the Master of Medical Research program. From the first cell culture workshop to final data analysis, you have consistently shown remarkable dedication and professionalism. It has been a pleasure working with you.

To fellow members of the Rose'Meyer lab group, Mr. Julian Lang-Lemckert, Ms. Emiri Tarbit, and Ms. Tamara Palmar, thank you for always being approachable and helpful.

Finally, thank you to my partner, Cat, to whom I owe everything.

In loving memory of Dr Gemma Diessel.

Abstract

Introduction

Pregnancy-related disorders such as pre-eclampsia (PE), intrauterine growth restriction (IUGR), and pre-term birth (PTB) significantly contribute to maternal and fetal morbidity and mortality in all human societies. Each of these conditions can result from placental insufficiency, a pathophysiological condition affecting the placenta due to trophoblast dysfunction. Maternal complications during pregnancy such as gestational diabetes mellitus (GDM) and perinatal depression (PD) show significant associations with these placental pathologies. Perturbations in stress and inflammatory mediators, cortisol and TGF-β1, as well as the serotonin transporter of the serotonin system, have been associated with abnormal properties of trophoblast function including proliferation, migration, invasion, and remodelling which underpin trophoblast dysfunction and incomplete placentation. While glucocorticoid signalling, TGF-β1 signal transduction, and the serotonin system have been implicated in the aetiologies of these pathological conditions, their interactions have been seldom explored in placental cell models.

Experimental aims

The aim of the present study was to investigate the effects of stress and inflammatory mediators, cortisol and TGF-β1, on cell metabolic activity and cytotoxicity measured using the MTT and LDH assays, protein expression of glucocorticoid receptor isoforms and the serotonin transporter measured using Western blot analysis, as well as mRNA expression of the glucocorticoid receptor measured using NanoString nCounter analysis in placental cell lines, SWAN-71 and JEG-3. The purpose of these experiments was to elucidate the relationships between stress, inflammation, and the serotonin system in

trophoblast pathophysiology and possible involvement in the aetiologies of PE, IUGR, and PTB.

Methods

SWAN-71 and JEG-3 cell lines were chosen as placental cell models for the present study. MTT and LDH assays were carried out to determine the effects of the proposed pharmacological treatments – hydrocortisone, mifepristone, TGF-β1, and SB431542 – may have on cell viability. Cells were treated with hydrocortisone (500 nM and 1 μM), mifepristone (10 nM), TGF-β1 (5 ng/mL), or SB431542 (1 μM) for a period of 24 hours; following which whole-cell lysates were extracted for subsequent protein or mRNA studies. Extracted protein was first quantitated using the BCA assay and then glucocorticoid receptor isoforms (GRα-A, GRβ, GRγ, GRP, GRA, GRα-C, and GRα-D) and serotonin transporter proteins were semi-quantitated through Western blot analysis. The A549 cell line was used as the internal standard as well as the positive control for both proteins; and cofilin was used as a loading control for all wells. Extracted mRNA was quantitated using NanoString nCounter techniques where total glucocorticoid receptor mRNA were measured. All data were presented as mean ± standard deviation. Cell viability assay and Western blot data were analysed via one-way ANOVA with Tukey's post-hoc comparison of means; while NanoString nCounter data were analysed via unpaired student T-test with Welch's correction. These statistical analyses were performed using GraphPad Prism Version 8.0, with a cut-off $p < 0.05$ for statistical significance.

Results

MTT and LDH viability assays showed that hydrocortisone and TGF-β1 treatments did not affect overall cell viability in either SWAN-71 or JEG-3 cells after 24 hours ($p > 0.05$). Western blot analysis revealed that both hydrocortisone and TGF-β1, in isolation

and in combination, downregulated GRα-A isotype protein expression in both SWAN-71 and JEG-3 cells after 24 hours with no additive effects observed when combined ($p < 0.05$). Further, TGF-β1 also upregulated GRβ protein expression in JEG-3 cells ($p < 0.05$). In SWAN-71 and JEG-3 cells treated with hydrocortisone or TGF-β1, individually and in combination, there was significant upregulation of SERT protein expression after 24 hours with no additive effects observed when combined ($p < 0.05$). Moreover, hydrocortisone and TGF-β1 had no effect on SWAN-71 cell total GR mRNA expression after 24 hours through NanoString nCounter analysis. Mifepristone and SB431542 had no effect on GR isotype nor SERT protein expression after 24-hour treatments in both SWAN-71 and JEG-3 cell lines ($p > 0.05$). SB431542 also had no effect on total GR mRNA expression after 24-hour treatments in SWAN-71 cells ($p > 0.05$).

Conclusion

The present study was the first to investigate the effects of stress and inflammatory mediators on cell viability, GR isotype and SERT protein expression, as well as total GR mRNA expression in the placental cell lines, SWAN-71 and JEG-3. Novel findings included the downregulation of GRα-A and upregulation of SERT protein expression after treatment with hydrocortisone or TGF-β1, individually and in combination, with no additive effects in the placental cell lines, SWAN-71 and JEG-3. TGF-β1 was also observed to upregulate GRβ in JEG-3 cells. These results indicate that perturbations in stress and inflammatory physiology can have deleterious effects on both GR and serotonin signalling processes that can alter trophoblast function and play a role in the aetiologies of pregnancy-related pathologies such as PE, IUGR, and PTB.

Statement of originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published nor written by another person except where due reference is made in the thesis itself.

_____

Dylan Robertson 27th February 2019

Table of contents

List of figures

CHAPTER 2

CHAPTER 3

List of abbreviations

5-HIAA	5-hydroxyindoleacetic acid
5-HT receptor	Serotonin receptor
11β-HSD	11β-hydroxysteroid dehydrogenase
ACTH	Adrenocorticotropic hormone
AF	Activation function domain
ALDH	Aldehyde dehydrogenase
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BCA	Bicinchoninic acid
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CBG	Corticosteroid-binding globulin
CHS	Cholesteryl hemisuccinate
CRH	Corticotropin-releasing hormone
CT	Cytotrophoblast
DAT	Dopamine transporter
DBD	Domain-binding domain
DMEM	Dulbecco’s modified essential medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco’s phosphate-buffered saline
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor

EPO	Erythropoietin
EVT	Extravillous trophoblast
FBS	Fetal bovine serum
GC	Glucocorticoid
GDM	Gestational diabetes mellitus
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HIF-1α	Hypoxia inducible factor 1-alpha
HPA	Hypothalamic-pituitary-adrenal
hsp	Heat shock protein
IFN-γ	Interferon-gamma
IL	Interleukin
IUGR	Intrauterine growth restriction
LBD	Ligand-binding domain
LDH	Lactate dehydrogenase
LIMK1	LIM kinase 1
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
mRNA	Messenger ribonucleic acid
miRNA	Micro ribonucleic acid
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
NAG	N-acetylglucosamine
NET	Noradrenaline transporter
nGRE	Negative glucocorticoid response element

NR3C1	Nuclear receptor subfamily 3 group C member 1
NSS	Neurotransmitter sodium symporter
NTD	Amino terminal domain
PAR6	Partitioning defective 6
PD	Perinatal depression
PE	Pre-eclampsia
PI3K	Phosphoinositide 3-kinase
PMAT	Plasma membrane monoamine transporter
PTB	Pre-term birth
PVDF	Polyvinylidene difluoride
SERT	Serotonin transporter
SLC6A4	Solute carrier family 6 member 4
SSRI	Selective serotonin reuptake inhibitor
ST	Syncytiotrophoblast
SUA	Spiral uterine artery
TBS	Tris-buffered saline
TBST	Tris-buffered saline with tween
TGF-β	Transforming growth factor-beta
TGF-βR	Transforming growth factor-beta receptor
Th	T-helper cell
TNF-α	Tumour necrosis factor-alpha
TPH	Tryptophan hydroxylase
TRIM33	Tripartite motif containing 33
VMAT	Vesicular monoamine transporter

Contributions

I, Dylan Robertson, hereby testify to the contributions made by myself and others in the production of this thesis:

Mr. Dylan Robertson

- Maintained all cell cultures
- Performed MTT and LDH cell viability assays
- Performed all drug treatments
- Performed all protein extractions
- Performed all cell lyses for protein and nucleic extraction
- Performed all data collection, entry, and subsequent statistical analyses
- Authored thesis

Associate Professor Roselyn Rose'Meyer

- Conceived, planned, and supervised project
- Made up stock solutions of all drugs
- Refined research proposal and write-up

Dr. Jelena Vider

- Assisted with cell culturing training
- Provided cell culturing and western blotting advice
- Assisted with the NanoString nCounter® experimental protocol

Mr. Samuel Hall

- Assisted with cell culturing, BCA assay, and western blotting technical training

CHAPTER 1: Literature review

1.1 Introduction

Maternal stress, inflammation, and the serotonin system have each been directly linked to several pregnancy-related pathologies such as pre-eclampsia (PE), intrauterine growth restriction (IUGR), pre-term birth (PTB), and, to a lesser extent, maternal conditions such as gestational diabetes mellitus (GDM) and perinatal depression (PD). PE is regarded as a multisystemic pregnancy-related hypertensive disorder and the primary cause of maternal and fetal morbidity and mortality, affecting 3-5% of pregnancies worldwide (1, 2). Once diagnosed, the prognosis often includes termination of the pregnancy or maternal mortality due to eclamptic complications during labour. Intrauterine growth restriction affects up to 15% of pregnancies and is predominantly defined as failure of the fetus to reach their genetic growth potential in utero with significant short- and long-term outcomes for the baby (3-9). PTB, defined as a birth occurring at less than 37 weeks gestation, is often secondary to maternal or fetal complications and similarly a major factor contributing to neonatal mortality (10). Surviving children may also experience various short- and long-term health defects such as neurodevelopmental and cognitive disorders, sensory impairments, motor disorders, risk of severe infections and long-term metabolic, cardiovascular, and mental health disorders (11). There are approximately 15 million PTBs that occur worldwide each year (12), with subsequent complications accounting for 15% of all child deaths and 35% of all neonatal deaths (13, 14). With respect to maternal risk factors, GDM is a condition that affects around 4-18% of pregnant women (15-17) and is often accompanied by fetal complications such as macrosomia, predisposition to platelet hyper-aggregation, congenital malformations, and increased risk of perinatal mortality

(18). Moreover, PD is a condition affecting approximately 10% of pregnancies and refers to depressive episodes during pregnancy or within the first 12 months postpartum (19-21). Depression during gestation has been associated with an increased incidence of PTB, low birth weight, fetal growth restriction, postnatal complications (22), as well as negative outcomes such as social isolation and marital discord for the mother (23, 24). These gestational pathological conditions are seemingly related and result in significant human suffering in the form of maternal and fetal morbidity and mortality, and also burden healthcare economies worldwide (25-29).

As briefly mentioned, stress, inflammation, and serotonin physiology – with particular respect to glucocorticoid transcriptional regulation, TGF-β signal transduction, and serotonin transporter activity – appear to play key roles in the immune-endocrine interactions that ultimately modulate numerous responses to environmental perturbations that may occur during gestation. However, these interactions remain largely undefined, especially in the context of placental pathophysiology. Herein lies the original and significant contribution to knowledge: the present study aimed to elucidate potential interplay between glucocorticoid, TGF-β, and serotonin system mechanisms in trophoblasts to refine our current understanding of their involvement in the aetiology of pregnancy-related pathologies. Subsequent findings will give rise to more sophisticated research that may ultimately lead to diagnostic, treatment, or preventative advances that could attenuate the morbidity and mortality of pregnancy-related pathologies.

1.2 Placentation and trophoblasts

The placenta is a multifunctional organ that is essential for fetal development and survival (30). The early developmental stages of the placenta, namely between three and five weeks of gestation, remain largely unknown due to the ethical limitations

associated with sample collection (31). Due to artificial reproductive advances and extensive research in animal models, however, some knowledge of pre-implantation events has been elucidated. At approximately 7-9 days after fertilization, the blastocyst implants in the uterine wall where the outer trophectoderm layer penetrates between the luminal epithelial cells, invade the basal lamina and infiltrates deep into the uterine decidua (31). Subsequent restoration of the luminal epithelium contains both the embryo and placenta within the uterine wall (31). These developmental events are critical for a healthy pregnancy to proceed. Specialised cells derived from the trophectoderm, known as trophoblasts, provide the structural and functional components of the placenta and mediate the interactions between the mother and fetus at the maternofetal interface (32, 33). Three major subpopulations of trophoblast cells have been characterised in the human placenta and include the undifferentiated and proliferative cytotrophoblast (CT) that can give rise to either the syncytiotrophoblast (ST) or the extravillous trophoblast (EVT) (30, 31, 33). STs are fused CT cells that form a layer which lines the maternal blood sinusoids and is involved in exchange, absorption, and other endocrine functions (33). EVTs, on the other hand, arise as highly proliferative and migratory cell columns at the base of the anchoring villi and invade either the decidualised endometrium, becoming interstitial EVTs, or the spiral uterine arteries, becoming endovascular EVTs (33). Endovascular EVTs exhibit an endothelial phenotype that remodels the arterioles from high-resistance muscular low-flowing tubes into low-resistance non-muscular high-flowing tubes that adequately nourish the developing fetus (33, 34).

1.3 Placental pathologies and fetal outcomes

Compromised trophoblast differentiation, proliferation, invasion, and endovascular remodelling have been directly implicated in the pathogenesis of PE of the mother, IUGR and subsequent PTB of the fetus, and, to a lesser extent, associated with the pathophysiology of GDM and PD.

1.3.1 Pre-eclampsia

PE is an enigmatic and devastating gestation-specific syndrome and a major cause of maternal and fetal morbidity and mortality that affects 3-5% of all pregnancies – up to 20% in developed countries (1, 2, 36). Contemporary understanding is that PE manifests as a result of failed trophoblast transition from a proliferative to an invasive phenotype and inadequate remodelling of spiral uterine arteries (SUAs) into capacitance vessels (1, 37). This process allows for the adequate supply of blood and nutrients to the developing fetus and is regulated in part by environmental oxygen pressure. Physiologically, the placenta is low in oxygen, approximately 20 mmHg, whereas the maternal decidua approaches 70 mmHg (1). Decreased oxygen induces trophoblast proliferation, represses invasion, and protects DNA from oxidative damage (38). As EVTs proliferate toward the decidualised endometrium, into higher oxygen content, their invasive potential is gradually enhanced (38). The pathogenesis of PE is ostensibly regarded as a two-stage process: first, trophoblast invasion and endovascular remodelling are compromised; second, the manifestation of fibrosis (39). Fibrosis is a pathological scarring process, mediated by fibroblasts, associated with excessive deposition of extracellular matrix (ECM) in connective tissue that can lead to organ dysfunction (39, 40). The sustained pathological ischaemic environment as a consequence of improper endovascular remodelling, along with maternal chronic

inflammation, induce over activation of fibroblast cells that can result in the impaired placenta releasing pathogenic mediators into the maternal circulation which induce endothelial dysfunction, perturbed coagulation, and hypertension (1, 38). Another feature in pre-eclamptic pregnancy is excessive trophoblast deportation, an otherwise normal phenomenon first described in 1893, where membrane-enclosed multinucleated structures named syncytial knots are extruded into maternal circulation and deported from the uterus (41-45). Syncytial knots are composed of apoptotic syncytiotrophoblast cellular debris in the form of micro- and nanoparticles, as well as mononuclear trophoblasts (45). If apoptotic cells are not removed, secondary necrosis can take place leading to an increased inflammatory response which would contribute to the adverse gestational milieu (45). Generally PE is only clinically diagnosed after hypertension together with evidence of systemic disease, often proteinuria, are observed in pregnant women (1). The severity of PE is dependent upon symptom onset, where early PE is defined as less than 34 weeks gestation and poses a high risk to fetal mortality and premature delivery as well as the progression to eclampsia for the mother, a potentially fatal condition characterised by seizures (1, 2). It is therefore essential to improve our understanding of the risk factors and underlying physiology to accurately diagnose, treat, or even prevent early PE.

1.3.2 Intrauterine growth restriction

IUGR is defined as a condition in which the fetus exhibits poor growth and fails to reach its genetic growth potential *in utero* (4). While various parameters have been set, fetal growth less than the 10^{th} percentile for gestational age detected via ultrasound is commonly cited for diagnosis (8, 9). IUGR affects approximately 10-15% of pregnancies, depending on geographic location as well as population and is associated with a high incidence of perinatal morbidity and mortality (3, 4, 8, 9, 46). For instance,

IUGR is associated with pre-term delivery, intrapartum asphyxia, respiratory distress, intraventricular haemorrhage, hypoglycaemia, hypocalcaemia, polycythaemia, necrotizing enterocolitis, maldevelopment of the fetal brain and metabolism, or even intrauterine fetal death (3-7). Interestingly, IUGR aetiology remains poorly understood due to the presence of significant heterogeneity. According to the literature, a timing of the insult during gestation may determine whether IUGR is symmetric or asymmetric which refers to the weight, length, and head circumference of the fetus: the former is characterised by each parameter being less than the 10^{th} percentile while the latter only pertains to birth weight (9). The causes of symmetric IUGR include genetic disorders and syndromes, infections, or intrauterine drug exposures. In contrast, asymmetric IUGR is often associated with impaired uteroplacental function or placental insufficiency (8, 9, 47). A recent review identified that a relative decrease in placental mass and function could result in fetal growth impairment, with studies highlighting abnormal placental implantation as a significant risk factor (4, 48, 49). In addition, increased syncytiotrophoblast apoptosis has been observed in cases of IUGR (50). Findings such as these signify the pertinence of trophoblast pathophysiology to IUGR pathogenesis.

1.3.3 Pre-term birth

PTB is characterised by delivery before 37 weeks gestation and affects up to 15 million pregnancies each year (10, 12). Surviving pre-term neonates may experience severe neurodevelopmental and cognitive disorders, sensory impairments, motor disorders, risk of severe infections and long-term metabolic, cardiovascular, and mental health disorders (11). As PTB occurs secondary to maternal or fetal complications, both PE and IUGR are significant risk factors, where the common denominator appears to be placental insufficiency. In the literature, inadequate placental supply is implicated in

PTB aetiology with placental pathology exhibiting remarkable negative and positive predictive value (51). Abnormal uteroplacental nutrient transport has similarly been attributed to the previously described inadequate SUA remodelling by trophoblasts and found to contribute to placental tissue damage and adverse pregnancy outcomes, including PTB (51-53). Therefore, if placental malperfusion is the underlying cause of PTB, then contemporary research would suggest that maternal obesity, geographic altitude, and the fetoplacental demands of twins, molar pregnancies, or larger sized fetuses (present in gestational diabetes) are all important risk factors (51).

1.4 Maternal complications associated with pregnancy

1.4.1 Gestational diabetes mellitus

GDM is a reversible pregnancy-related condition defined as glucose intolerance with onset or first recognition during pregnancy that affects around 4-18% of pregnant women (15-17). During pregnancy, there is greater insulin demand on the mother due to the increasing nutrient requirements of the developing fetus (54), inducing proliferation of pancreatic β cells (55), a decrease in glucose threshold (56), and an increase in glucose-stimulated insulin secretion (57). If insulin production fails to meet the insulin demand, GDM manifests (58). In general, women who develop GDM are older, overweight, have higher blood pressure, have a higher proportion of large for gestational age fetuses, and have an increased risk of developing type 2 diabetes later in life (16, 59). GDM has also been associated with impaired trophoblast proliferation, invasion, implantation, villous differentiation, and placental mass expansion which indicates that there is interference with normal trophoblast physiology that may contribute to the pathogenesis of PE and PTB (60). While GDM may appear to be unrelated to PE due to differing clinical presentations and diagnostic criteria, many

studies have drawn distinct correlations between the two conditions such as perinatal outcomes and common risk factors including maternal age, nulliparity, and high pre-pregnancy body mass index (61-63). The extent to which GDM affects placental functionality and precipitates other pregnancy-related pathologies remains to be elucidated.

1.4.2 Perinatal depression

PD is a condition affecting approximately 10% of pregnancies and is characterised by major or minor depressive episodes during pregnancy or within the first 12 months postpartum (19, 20). Depression during gestation has been associated with an increased incidence of PE, IUGR, PTB, and low birth weight, as well as negative outcomes such as social isolation and marital discord for the mother (22-24). Interestingly, contemporary research in this field has premised the phenomenon of fetal programming, the existence of a relationship between abnormal maternal neuroendocrinology, as observed in mental illness, and subsequent emotional, behavioural, cognitive, stress physiology, immune function, and metabolic maldevelopment in the offspring (64-67). More specifically, aberrations in stress and inflammatory physiology have been postulated to be the predominant models in the pathogenesis of depression and, consequently have also been hypothesised as mechanisms for biologically embedding fetal programming (67-70). Furthermore, a logical extension of the perturbed stress and inflammation biochemistry hypotheses would implicate roles in trophoblast physiology and placental organ functionality.

Due to the complex nature of pregnancy-related pathologies, studies have only detailed various associations and inherently lack explanatory power with respect to underlying physiological processes. Nonetheless, the evidence that has been elucidated, albeit

circumstantial, indicates that relationships certainly exist and warrant further investigation.

1.5 The stress response

Stress is defined as a state of real or perceived threat to homeostasis that may challenge an organism's well-being (71). To restore homeostatic conditions, a complex range of responses involving the endocrine, nervous, and immune systems – collectively known as the stress response – are activated to prioritise immediate survival over less essential physiological functions (72, 73). The hypothalamic-pituitary-adrenal (HPA) axis is the principal effector of the stress response, which triggers the paraventricular nucleus of the hypothalamus to release corticotropin-releasing hormone (CRH) and arginine vasopressin which stimulate the production of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. In the adrenal glands, ACTH induces the synthesis and secretion of the primary stress glucocorticoid (GC), cortisol, from the zona fasciculata, as well as the mineralocorticoid, aldosterone, and other adrenal androgens (74). The stress response is terminated when rising levels of cortisol inhibit further release of CRH and ACTH in a negative feedback loop allowing a return to a homeostatic physiological state (75).

1.6 Glucocorticoids and the glucocorticoid receptor

GCs are lipid-soluble hormones synthesised in the adrenal cortex that mediate a multitude of physiological functions (76). GCs are essential regulators of metabolism, the cardiovascular system, cell proliferation and survival, growth, cognition and behaviour, immune function, and reproduction (77). GCs are also frequently utilised for clinical applications as anti-inflammatory and immunosuppressant drugs (75). Following their release from the adrenal glands, GCs are transported through the blood

in an inactivated state bound to corticosteroid-binding globulin (CBG) and albumin (75, 76). Upon reaching their target destination, GCs are released from their protein carriers to bind to intracellular glucocorticoid receptors (GRs) where all of the cellular and pharmacological actions of the GCs are mediated (75). The GR is the founding member of the nuclear receptor superfamily of ligand-dependent transcription factors that is expressed in nearly all human cells and responsible for regulating thousands of genes unique to the cell type (75, 78). Closely related to its paralogues, the mineralocorticoid receptor, progesterone receptor, and androgen receptor, GR is encoded by the nuclear receptor subfamily 3 group C member 1 (NR3C1) gene and possesses a modular structure comprised of three distinct functional domains: an amino-terminal transactivation domain, a central DNA binding domain, and a carboxy-terminal ligand - binding domain (75, 79). An interesting feature of the GR is a flexible hinge region located between the DNA-binding domain (DBD) and the ligand-binding domain (LBD) provides structural versatility for genomic interactions and contains a nuclear localisation signal (75). Furthermore, embedded within these functional domains are the activation function domain 1 (AF1), tau2, and AF2, which are responsible for regulatory activity (79).

The GR actively shuttles between the cytoplasm and nucleus in response to ligand binding. In the absence of cortisol, the endogenous ligand, or synthetic GCs, such as hydrocortisone or dexamethasone, monomeric GR resides predominantly in the cytoplasm as part of a large multi-protein complex that includes chaperone proteins, heat shock protein (hsp) 90, hsp70, and p53, as well as immunophilins of the FK06 family (75, 77, 80). Upon ligand binding, a conformational change is induced that results in GR translocation and accumulation in the nucleus, allowing for subsequent interactions with specific genomic loci and other transcription factors (75). More

specifically, binding at DNA sequences are termed GR response elements (GREs) where the remodelling of chromatin, the recruitment of co-regulators, and the initiation of transcription is stimulated (75, 81). GRs can also bind negative GREs (nGREs) which leads to transcriptional repression through the recruitment of co-repressors (75, 81). Moreover, GRs are also able to mediate gene transcription through interactions with other transcription factors with or without contacting DNA (75). The N-terminal AF1 domains and surrounding NTD regions exhibit variation in both sequence and size that, along with alternative splicing of exons and translational start sites, can give rise to multiple GR isoforms which control common and unique genes with distinct cellular localisation patterns in response to GC ligand binding (79). More specifically, exons 2-9 encode for the DBD, hinge region, and LBD of the GR and undergo alternative splicing that results in transcripts and subsequent proteins, GRα, GRβ, GRγ, GRA, GRP (82-85). For instance, the two most studied isoforms of GR, GRα and GRβ, are identical up to amino acid 727, at which point they diverge: GRα has an additional 50 amino acids in the LBD while GRβ only possesses 15 (86). As a consequence, GRα and GRβ have distinctly different effects on gene transcription (79, 86). Generally, GRα is primarily found in the cytoplasm and translocates to the nucleus upon ligand binding (87), while the transcriptionally-inactive GRβ resides in the nucleus with inhibitory effects on GRα –mediated transcriptional activity (85, 86, 88-91). Furthermore, GRα messenger ribonucleic acid (mRNA) can generate alternative N-terminal isoforms as a result of alternative translation initiation sites to form GRα-A, GRα-B, GRα-C1, GRα-C2, GRα-C3, GRα-D1, GRα-D2, and GRα-D3 isoforms (82). These various isoforms of the GRα protein exhibit unique activities, with the GRα-D isoforms residing in the nucleus while the GRα-A, -B, and –C isoforms are localised primarily in the cytoplasm and only undergo nuclear translocation after ligand binding (85, 92, 93).

Essentially, the extent to which specific cells respond to GCs depends upon the ligand type and concentration, post-translational modifications to GR, relative abundance of co-regulators or co-repressors, the chromatin environment and the GR-bound DNA sequence involved (75, 79). Interestingly, GRs paradoxically display both precision and plasticity with respect to context-specific gene transcription regulatory activity and overarching function of cell type, with vastly different effects between cells (79). As such, the dynamics of the GR remain poorly understood.

1.7 Placental glucocorticoid signalling

GCs have essential biological roles in human physiology as well as preparing the fetus for the extrauterine environment (85, 94, 95). Hypercortisolaemia is a normal physiological event during pregnancy that intensifies over the course of gestation (96). Literature has shown that high levels of cortisol are crucial for the development and maturation of various fetal tissues, such as neurological, cardiovascular, respiratory, gastrointestinal, hepatic, renal, as well as haematopoietic and lymphatic systems (94, 97, 98). While the maternal HPA axis does become hyperactivated during pregnancy (85), the otherwise supraphysiological amounts of circulating cortisol are also a consequence of a positive feedback mechanism due to the synthesis and release of CRH and ACTH by the placenta (99-102). The steroid hormones progesterone and oestrogen are upregulated and have marked effects on glucocorticoid physiological activity (85). Progesterone binds to CBG with higher affinity than cortisol (103), resulting in a reduction in total bound cortisol while rising oestrogen leads to a doubling of CBG levels that effectively lower catabolism of cortisol by the liver, doubling cortisol half-life in plasma (104). Remarkably, maternal cortisol levels are 5- to 10-fold higher than in the fetus (105-109), a gradient strictly regulated by placenta 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes (85, 106). In placental mammals, 11β-HSD2,

highly expressed in the placenta, converts active cortisol to inactive cortisone, acting as a “protective barrier” (110-113). Physiological and psychological events such as stress (114), malnourishment (115, 116), depression (117), obesity (118), or GC medications (119), can have profound effects on maternal GC levels, as well as saturate or downregulate 11β-HSD2 activity (95, 120, 121), leading to abnormally high maternofetal GC transfer. The transient exposure to excess GCs has been shown to reduce both GR mRNA and protein expression in a time- and dose-dependent manner that was reversible upon return to normal physiological conditions (92, 93). In chronic cases of elevated GC exposure, however, this does not appear to be the case and suggests that persistent exposure to excess GC may elicit more permanent aberrations in glucocorticoid signalling and subsequent regulatory physiological processes to the detriment of both the mother and fetus (85, 93, 122). With respect to GR expression in trophoblasts, several isoforms were identified in fractionated protein extracts from human placental tissue (122). Specifically, bands were detected that correspond to GRα (94 kDa), GRβ (91 kDa), GRα-C (81 kDa), GR-P (74 kDa), GR-A (65 kDa), and GRα-D1-3 (50-55 kDa), noting that GRα-D and unknown 38 and 48 kDa isoforms accounted for over 60% of the total GR expression in the placenta (122). A review investigating the regulation and physiological relevance of the many GR variants in the placenta, suggested a novel mechanism involving the varied responses to GCs in pathological placentae and hypothesised that placental pathophysiology may be mediated through altered responsivity to GCs via the expression of agonistic or antagonistic isoforms that do not mediate the putative effects of GCs (123). This also appears to be a phenomenon that is further complicated by differences in gestational age and fetal sex (123, 124). For example, male pre-term placentae have increased cytoplasmic levels of GRα-C and GR-A compared to term placentae, whereas female pre-term placentae exhibited higher

levels of nuclear GRα-C compared to term placentae (123). The literature has shown that GRα-C is a potent activator of the glucocorticoid-induced apoptosis and may play a significant role in the pathophysiology of pre-term placentae (122, 123). Beyond the genome, epigenetic changes such as methylation of the GR gene promoter have been associated with maternal smoking, stress, birthweight, and fetal neurodevelopment (123, 125-129). It is also worth noting that extensive research has been carried out in patients diagnosed with Cushing's syndrome, a condition characterised by chronically elevated cortisol levels, where incidences of GC resistance have been reported through increased GRβ expression accompanied by reduced GRα ligand binding affinity in peripheral mononuclear cells (130). These pathological changes were reversed after treatment with a return to the normal phenotype. In summary, perturbations in circulating GC levels may regulate GR variant isotype expression, in a reversible manner, with profound consequences in trophoblast function.

1.8 Maternal stress and excess glucocorticoid maternofetal transfer

As previously described, maternal blood plasma cortisol increases during the third trimester of pregnancy in excess of 420 nM, which is 2- to 4-fold higher than in non-pregnant women (109, 131-136). While cortisol is essential for normal fetal development (137, 138), excessive concentrations, as observed in prenatal maternal stress, have been linked to intrauterine growth restriction (139, 140), premature delivery (141, 142), fetal low birth weight (143, 144), and even fetal programming of adult pathophysiology in offspring (145). Maternal stress during the first trimester of pregnancy has been shown to increase the risk of PE through placentation disruption (146), with evidence of abnormal GC signalling (147). Placental cortisol levels have been observed to be higher in cases of PE relative to controls while maternal serum cortisol levels showed no statistically significant difference (148). Dexamethasone, a

synthetic form of cortisol, has been demonstrated to inhibit the expression of trophoblast invasion-related genes, matrix metalloproteinase (MMP)-2, MMP-9, and osteopontin, and develop PE-like phenotype in pregnant rats (149). In SWAN-71 cells, a modest decrease in trophoblast invasion was also observed (150). These findings were corroborated by microarray analysis reporting that dexamethasone inhibits trophoblast proliferation, migration, and invasion through the regulation of the *SERPINE1* gene (151). While it would appear that excess cortisol is associated with PE, the mechanisms involved have been seldom investigated. PTB has been associated with stress and negative emotional responses such as anxiety, depression and distress in many studies (152). Under normal conditions, the physiological stress response also involves alterations in immune function (153). Specifically, pro-inflammatory cytokines stimulate the HPA axis to secrete cortisol which down-regulates the pro-inflammatory cytokines in a negative feedback mechanism (152), and induces the production of anti-inflammatory cytokines (154). During chronic conditions, such as prenatal maternal stress, however, cortisol becomes less effective at suppressing immune function and can lead to subsequent increased susceptibility to infection and inflammation (155). Albeit several limitations, such as small sample sizes and varying types of cortisol samples at differing times of the day with associated recall bias, studies have indicated that higher levels of perceived stress are linked to elevated levels of pro-inflammatory cytokines and decreased levels of anti-inflammatory cytokines (152), along with stress being associated with shorter gestational length (156). Excess cortisol has also been associated with GDM as serum and urinary levels were found to be significantly higher in affected women compared to healthy controls (157) and women with the greatest increase in cortisol levels have also exhibited the greatest deterioration in glucose tolerance (158), however sample sizes in these studies were also small. Similarly, supraphysiological

cortisol appears to increase the vulnerability to developing depression (19) with several studies linking hypercortisolaemia to PD (159-162). In contrast, while studies have also found insignificant associations between excess cortisol and depression (163-173), a recent review identified limited sampling, insufficient statistical power, as well as cortisol determination methods as major limitations (174).

1.9 Inflammation and pregnancy

Another key process in the maintenance of homeostasis is inflammation, which involves the response of tissue to various insults (175, 176). It is characterised by the upregulation of cytokines and pattern recognition receptors that detect and react to the presence of microbes and products of tissue breakdown (176). Uncontrolled inflammation and deregulated cytokine networking during pregnancy can give rise to a many of the previously described adverse health outcomes for both the mother and fetus, with studies identifying pathogenic links to PE, PTB, and IUGR (177). During gestation, the innate immune system must be tightly regulated to prevent the rejection of the fetal allograft. Cytokine production is central to this process where various maternal and placental hormones exhibit potent immunomodulatory activity (176). For instance, progesterone has been shown to modulate antibody production, decrease monocyte activity, reduce proinflammatory cytokine production by macrophages, alter cytokine secretion of T-cells, and demonstrate protection against pre-term delivery (176, 178-180). Oestrogens appear to augment some immune responses, with modulatory effects exhibited through the inhibition of T-helper cell type 1 (Th1) proinflammatory cytokines, such as interleukin (IL)-12, tumour necrosis growth factor-alpha (TNF-α), and interferon-gamma (IFN-γ) while stimulating Th2 anti-inflammatory cytokines, IL-10, IL-4, and transforming growth factor-beta (TGF-β) (176, 181, 182). These immune-

endocrine interactions that ultimately modulate numerous responses to environmental perturbations during gestation remain largely undefined (176).

1.10 TGF-β

TGF-β, like other inflammatory mediators, has key regulatory roles during normal pregnancy, and disruptions in its expression and signalling have been associated with a range of gestational pathological conditions. TGF-β family members are pleiotropic cytokines with multitudinous cellular- and environmentally-dependent effects on immune functions, with regulatory and inflammatory activity (183, 184). TGF-β cytokines are initially synthesised as precursor molecules of TGF-β homodimers containing a propeptide region (185). This precursor interacts with a protein gene product from the N-terminal region, latency associated peptide, forming the small latent complex (186). This complex is then bound by latent TGF-β-binding protein, forming the large latent complex, which is released into the extracellular matrix in an inactive state (187). This complex remains inactive until properly processed by particular cellular cascades as required. TGF-β molecules bind to TGF-β receptor (TGF-βR) II or TGF-βRIII, triggering cytoplasmic kinase activity and ultimately activating TGF-βRI, which leads to downstream nuclear translocation of SMAD molecules and the subsequent transcription of target genes (184). Interestingly, in a manner similar to the cell type-dependent nature of GR signalling, TGF-β too exhibits contextual functionality (188). The TGF-β transcriptional response in a cell is shaped by three contextual determinants: the extracellular and intracellular composition of TGF-β signal transduction system, such as the abundance and activity of TGF-β ligands, receptors, and regulators; the factors that co-operate with SMAD proteins to regulate transcription, and; the epigenetic landscape of the cell, including DNA methylation, histone modification, nucleosome positioning, or non-coding RNAs that shape the chromatin

and determine which genes are open for expression and subsequently susceptible to regulation (188).

1.11 Placental TGF-β1 signalling

First cloned from human term placenta mRNA, TGF-β1 is the most abundant and widely expressed isoform (189, 190). In mesenchymal stem cells (MSCs) – existing in tissues such as placenta and umbilical cord – TGF-β1 stimulates proliferation via SMAD3-dependent nuclear accumulation of β-catenin (190, 191). Contrastingly, MSC proliferation can be inhibited by BMP2 antagonism of Wnt3a signalling via SMAD1/5 transduction (190, 192, 193). SMAD proteins appear to recapitulate the context-dependent nature of TGF-β action (190). TGF-β1 is also frequently heralded as the master regulator of fibrosis (194) and fibrotic changes are one of the most prominent features in the villous stroma of PE cases (39, 195, 196). As previously described, the first stage of PE pathogenesis is believed to be associated with compromised trophoblast invasion into the maternal decidua and endovascular remodelling (1, 37). Thereafter, placental chronic inflammation, vascular lesions, perivillous coagulation, and villous fibrosis are thought to follow (39). Fibrosis is a pathological scarring process associated with excessive deposition of extracellular matrix (ECM) in connective tissue, leading to organ dysfunction (39, 40). Fibroblasts mediate ECM maintenance and wound healing in connective tissue under normal physiological conditions and can be activated by TGF-β1 (197, 198). It is therefore hypothesised that excessive TGF-β signal transduction can lead to uncontrolled activation of placental fibroblasts, resulting in the fibrotic phenotype observed in pre-eclamptic placentae (39, 199). During pregnancy, TGF-β1 plasma levels are much higher compared to healthy non-pregnant controls (200) and literature has shown that TGF-β1 is ubiquitously expressed in trophoblast cells with dynamic involvement in cell proliferation,

differentiation, apoptosis, and remodelling (201). TGF-β1 has been shown to play a key regulatory role, particularly in early gestation, by exerting anti-proliferative and anti-invasive effects in normal placental cells (202-205). TGF-β1 promotes proliferation and invasion in tumour cells (206, 207), although the underlying mechanisms by which this occurs remain largely unclear. In the JEG-3 cell line, TGF-β1 promoted invasion of trophoblasts by upregulating SMAD2 and SMAD3 transcription factors, as well as increasing the expression of MMP-2 and MMP-9 (208). During the switch from proliferative to invasive phenotype in trophoblasts, TGF-β1 appears to be significantly downregulated (201), which indicates that defects in this process may be involved in the aetiology of PE. Similarly, in PTB, TGF-β1 is also down-regulated, along with IL-10, IL-4, and IL-1 antagonist members, while pro-inflammatory cytokines are up-regulated (209). Down-regulation of TGF-β1 has also been demonstrated in PD studies, with significantly lower levels of serum TGF-β1 when compared to healthy controls (210-213). Contrastingly, serum and plasma levels of TGF-β1 appear to be elevated in diabetic rodent models and humans (214-216), with a study reporting that women with GDM had higher levels of serum TGF-β1 than healthy controls, although lower levels than patients with type II diabetes (217). Once again, similar to GR transcriptional activity, the complex and context-dependent nature of TGF-β signal transduction gives rise to functional ambiguity, especially in placental pathophysiology, and it remains to be seen whether trophoblasts, fibroblasts, or both, are responsible for the observed pathological changes (39).

1.12 Serotonin system and SERT

The serotonin system is vast and complex, comprising of at least 18 different subtypes of receptors, with a wide range of roles in human physiology (218). Serotonin, also known as 5-hydroxytryptamine, was first discovered in the late 1940s with modulatory activity in the central nervous system, as well as cardiovascular function, digestion, body temperature, endocrinology, and reproduction (219-221). Serotonin is synthesised from tryptophan via rate-limiting hydroxylation by tryptophan hydroxylase (TPH) enzymes into 5-hydroxytryptophan and finally decarboxylated into 5-hydroxytryptamine by aromatic amino acid decarboxylase (222). Serotonin is packaged into vesicles by vesicular monoamine transporter (VMAT) and, with lower affinity, dopamine (DAT), noradrenaline (NET), and plasma membrane monoamine transporters (PMAT) and released in a calcium ion-dependent manner (223). Physiologically-active serotonin interacts with G-protein-coupled receptors and ligand-gated ion channels, known as serotonin receptors (5-HT receptors), that are involved in both wider excitatory and inhibitory physiological processes (221). Serotonin action is terminated by monoamine oxidase (MAO) and aldehyde dehydrogenase (ALDH) enzymatic degradation to 5-hydroxyindoleacetic acid (5-HIAA) following re-uptake by the serotonin transporter (SERT), a member of the neurotransmitter sodium symporter (NSS) family, along with previously mentioned DAT and NET (221).

SERT is an oligomeric glycoprotein comprised of 630 amino acids with cytoplasmic amino (N)- and carboxyl (C)-termini, encoded by the solute carrier family 6 group A member 4 (SLC6A4) gene on chromosome 17q11.2., that span 12 transmembrane domains along the plasma membrane of expressing cells (224, 225). Its primary function is the sodium- and chloride-dependent reuptake of serotonin, thus terminating interactions with serotonin receptors (221, 226, 227). Despite being one of the most

extensively studied members of the NSS transporter family, SERT remains a rather enigmatic protein with respect to its regulation by transcriptional, translational, and post-translational modifications. SERT uptake functionality depends on its structure, folding, membrane trafficking, association with other proteins, and assembly on the plasma membrane. Contemporary theories suggest that these activities are regulated by post-translational modifications, such as glycosylation (228), phosphorylation (229), disulphide bond formation (230), and oligomerizations (231). The contributions of such modifications to SERT or how these modifications transform in pathology remains equivocal (225).

1.13 Placental serotonin signalling

Serotonin also plays an essential role during placentation and intrauterine fetal development. Trophoblasts have been demonstrated to express serotonin system components, including receptors 5-HT_{1A} and 5-HT_{2A} (232, 233), transporters SERT and monoamine oxidase (MAO) which terminate the physiological actions of serotonin (18, 234), as well as enzyme isoforms tryptophan hydroxylase 1 (TPH1) and 2 (TPH2) (235) that produce *de novo* serotonin from maternal L-tryptophan (236). While studies have concluded that serotonin synthesis in the placenta is the predominant source of fetal serotonin, immunocytochemistry studies in both mice and humans have found evidence to the contrary, instead concluding that maternal serotonin is delivered to the conceptus (237). In any case, serotonin plays a key mitogenic role in early embryogenesis and has been shown to enhance trophoblast cell viability and progression through the cell cycle (238, 239). Serotonin signalling must be tightly controlled as it mediates the growth, differentiation, and apoptosis of trophoblasts during the essential process of placentation (240). For instance, continuous serotonin signalling has been implicated in activating various cell death mechanisms (241-244), with many studies involving serotonin re-

uptake inhibitors (SSRIs) indicating that unregulated serotonin increases the rates of congenital prematurity and malformations (245-249). Contrastingly, tryptophan hydroxylase 1 knock-out studies have demonstrated decreased trophoblast implantation and pregnancy success rates (250). These findings highlight the crucial role that SERT plays in early gestation by regulating serotonin signalling, although under- or over-expression of SERT may exert a pathological outcome.

Serotonin was first proposed as an aetiological factor in PE in 1960 (251) and research has shown that during pregnancy and, to a greater extent in PE, serotonin levels are markedly elevated along with increased placental sensitivity to serotonin (252). The placentae of patients with PE exhibit significantly raised serotonin when compared to controls (253). Further, increased traces of serotonin and metabolites have also been observed in urinary excretion of patients with PE (254, 255). These findings indicate that abnormal serotonin metabolism and signalling processes during gestation may be involved in the pathogenesis of PE. Serotonin and SERT are similarly implicated in the pathogeneses of PTB, GDM, and PD. In a review, women receiving SSRIs during pregnancy had a markedly higher risk of PTB when compared to untreated women with depression and healthy controls (22). It should be noted that there were no randomised controlled trials included in the review, confounding variables were inconsistently controlled, and the severity of depression between cases potentially resulted in significant bias. With respect to GDM, studies have indicated that serotonin modulates glucose homeostasis and causes rapid glucose uptake in both L6 myotubes and isolated rat skeletal muscle via the 5-HT_{2A} receptor (256, 257). Activation of 5-HT_{2A} receptors has been shown to impair glucose uptake by interfering with insulin-induced capillary recruitment in skeletal muscle (258, 259). Although contradictory, it is clear that serotonin and 5-HT_{2A} receptor are involved, to some extent, in peripheral glucose

metabolism. Serotonin has also been shown to increase the sensitivity of maternal pancreatic β cells to glucose during pregnancy (54) and functions in a paracrine-autocrine manner to induce pancreatic β cell proliferation (241), with evidence of SSRIs inducing insulin resistance by directly inhibiting the insulin signalling cascade in pancreatic β cells (260). Contrastingly, the defective insulin signalling in GDM has been shown to hinder the dissociation of SERT from endoplasmic reticulum protein 44, a necessary process for SERT to perform proper posttranslational modifications and move to the trophoblast plasma membrane (261). Interestingly, serotonin signalling may even provide a significant connection between depression and both type II diabetes and GDM (262, 263). Finally, in PD, chronic or exaggerated activation of the stress response system has been shown to result in excessive secretion of stress hormones that directly increase the uptake of neurotransmitters such as serotonin, which may lead to depressive symptoms (264-267).

1.14 Glucocorticoids and SERT

Excess cortisol has been associated with various serotonin-deprived pathologies (268). In rodent and human experiments, stress and elevated maternal cortisol levels have been observed to upregulate SERT expression (269-271). Further, dexamethasone has been shown to increase the mRNA and expression of SERT protein in human B-lymphoblastoid cells (272) and was also demonstrated to rapidly increase SERT cell surface expression through GR-mediated mechanisms in neurons, *in vivo* (273). Moreover, miRNA are small non-coding molecules involved in post-transcriptional regulation of specific gene expression (274). An miRNA that targets and modulates SERT expression, miR-16 (275), has been identified in human placental cells (276). Literature has shown that when miR-16 is under-expressed, SERT expression increases (277). Interestingly, miR-16 is significantly decreased in low birth weight infants, with

studies linking excess cortisol and dexamethasone to target and decrease the expression of miR-16 (278, 279). Furthermore, epidermal growth factor (EGF) and EGF receptors have been identified in trophoblasts (280) and appear to play a role in cell proliferation and invasion (281). EGF has also been shown to increase SERT expression via EGF receptor activation in rodent intestinal epithelial cell studies (282) and cortisol has been demonstrated to increase EGF binding and EGF receptor expression in human endometrial cells (283). These findings suggest the possible existence of mechanisms for SERT over-expression as a consequence of excess cortisol in trophoblasts, with subsequent decrease in serotonin signalling and disruption of implantation. The switch in phenotype, from proliferative to invasive, occurs as a consequence of various complex signalling processes (1). According to literature, erythropoietin (EPO) and its receptor have been identified in trophoblast cells and their interaction plays a key role in trophoblast cell survival, proliferation, and differentiation (284-286). EPO is regulated by hypoxia-inducible factor 1-alpha (HIF-1α) and is expressed under hypoxic conditions, similar to that observed in early gestation (287). HIF-1α similarly regulates MMP-12, a highly expressed protease that is specific to endovascular trophoblasts and is suggested to be involved in trophoblast invasion (288). The activation of 5-HT_{1A} receptors have been demonstrated to inhibit HIF-1α expression in rodent embryonic serotonergic cells (289). However, whether decreases in trophoblast EPO or MMP12 are involved in the aetiology of PE, or if 5-HT_{1A} receptor activation inhibits HIF-1α in trophoblasts, remains to be seen. The SSRI fluoxetine has been shown to markedly increase the expression of miR-135 in rodent studies (290), suggesting that miR-135 downregulation might occur as a result of SERT over-expression. Previous studies have implicated miR-135 in the regulation of placental cell growth (291-293) and the promotion of tumourigenesis by targeting HIF-1α pathway in rodent studies (294).

Hypothetically, it is possible that a decrease in trophoblast serotonin activity, as a consequence of SERT over-expression due to excess cortisol, disrupts normal trophoblast proliferation and invasion, leading to pregnancy-related pathology.

1.15 TGF-β1 and SERT

Due to the complex and ubiquitous nature of serotonin and TGF-β signal transduction systems, relationships between these processes have garnered some attention in contemporary research with particular respect to SERT. For example, studies involving epithelial colorectal adenocarcinoma cell lines have revealed that TGF-β1 interacts with TGF-β1R in a manner that optimises SERT function by increasing exocytosis, cellular trafficking, and recruitment to the plasma membrane (295). TGF-β1 has been shown to upregulate SERT function and expression through PI3K and SMAD 3-mediated pathways (296). Contrastingly, TGF-β1, along with TGF-β3, were shown to up-regulate miR-424(322) and miR-503, but not miR-16, in rodent studies, indicating that TGF-β1 may have no effect on SERT via miRNA-mediated post-transcriptional modifications (297). Experiments have also reported anti-inflammatory effects of SSRIs which posit a corollary that decreased SERT activity is directly associated with reduced inflammation (298-301). Evidence of TGF-β1 and SERT interactions beyond mere correlation is certainly left wanting in the literature and merits serious investigation. Indeed, any measurable interactions present in trophoblast cell models would be novel.

1.16 TGF-β1 and GR

As previously described, TGF-β and glucocorticoid signalling pathways exhibit pleiotropic regulation in a variety of physiological and pathological processes. The effects of glucocorticoids on TGF-β responses have been thoroughly investigated in current literature. However, the influence that TGF-β transduction systems have on GR

transactivity has been seldom explored, especially in placental cell models. In murine fibrosarcomal L929 cells, TGF-β1 antagonised GR transactivity and inhibited GR-mediated gene expression after dexamethasone-mediated GR nuclear translocation (302). This study concluded that cross-talk between the two signalling systems could be explained in part by modulation of transcription factor AP-1 (302). Moreover, SMAD6, a downstream TGF-β1 inhibitory coding protein, silences GR-induced transactivation by complexing with histone deacetylase 3, and acts as a corepressor of the GR in rat liver tissue (303). TGF-β1 was shown to reduce the cellular levels and nuclear localisation of GRα-A, impairing glucocorticoid activity in lung adenocarcinoma A549 cells (304). Contrastingly, TGF-β1 has been demonstrated to increase glucocorticoid binding and signalling in inflammatory cells via SMAD2/3 and AP-1 mediated processes while another study indicated TGF-β1 and GRβ may co-operate in the pathogenesis of GC-resistance in nasal polyposis (305, 306). Given the remarkable plasticity and context-dependent nature of both TGF-β signal transduction and the GR, observations in different cells may provide very little indication of what might occur in trophoblast physiological and pathological conditions and warrants further investigation.

It is without question that significant advancements have been made in the field of early gestation trophoblast physiology; however, the specific mechanisms underpinning the aetiologies of many pregnancy-related pathologies remain elusive particularly with respect to stress, inflammation, and serotonin system signalling. The present study aimed to; 1. determine the effects of stress and inflammatory mediators, hydrocortisone and TGF-β1, on SWAN-71 and JEG-3 trophoblast cell line viability to assess the suitability of pharmacologically-treated placental cell models for future investigations (Chapter 2), and; 2. determine the effects of stress and inflammatory mediators,

hydrocortisone and TGF-β1, on GR isotype and SERT protein expression as well as total GR mRNA expression to clarify their potential roles in human trophoblast pathophysiology (Chapter 3). The contemporary research literature indicated that SWAN-71 and JEG-3 cell lines are frequently utilised trophoblast cell models and that hydrocortisone and TGF-β1 pharmacologically mimic stress and inflammatory conditions to an appropriate and pragmatic extent. It was therefore hypothesised that SWAN-71 and JEG-3 placental cell line treatment with hydrocortisone and TGF-β1 would be suitable models for future investigations. It was hypothesised that hydrocortisone and TGF-β1 would induce changes in both GR isotype and SERT protein expression in both SWAN-71 and JEG-3 cell lines. Finally, it was also hypothesised that there would be no change in total GR mRNA in SWAN-71 cells, as changes in individual GR isotype mRNA would not be accounted for.

The ultimate purpose of these experiments was to determine the effects of stress and inflammatory mediators on trophoblast physiology to elucidate the pathological mechanisms involved in the aetiologies of pregnancy-related pathologies such as PE, IUGR, and PTB. Subsequent findings may provide original and significant opportunities for further research as well as diagnostic, treatment, or preventative advances in the future to attenuate the maternal and fetal morbidity and mortality in clinical practice.

CHAPTER 2: SWAN-71 and JEG-3 cell metabolic activity and cytotoxicity after treatment with hydrocortisone or TGF-β1 for 24 hours

2.1 Introduction

Perturbations in stress and inflammatory mediators, cortisol and TGF-β1, have been implicated in pathological conditions such as PE, IUGR, and PTB as well as maternal complications GDM and PD (19, 140-144, 157-159, 162, 201, 209-217). Maternal cortisol and TGF-β1 levels have been shown to increase throughout gestation in a carefully controlled manner and is considered a normal physiological phenomenon (96, 200, 201). It is therefore critical to identify appropriate placental cell models to investigate these relationships *in vitro* to advance our understanding of how stress and inflammation may affect trophoblast function and contribute to the pathogeneses of PE, IUGR, and PTB.

SWAN-71 cells are immortalized first trimester trophoblasts via transfection with human telomerase reverse transcriptase (307). The SWAN-71 cell line has been demonstrated to retain the attributes and characteristics of primary first trimester trophoblasts after more than 100 passages and represents a valuable model for *in vitro* trophoblast studies (307). Another placental cell line, JEG-3, is a choriocarcinoma cell line and has been available for over 40 years with extensive research literature underpinning their use (308, 309). These cell lines have been widely cited in the literature as *in vitro* models for studies pertaining to trophoblast pathophysiology and utilised for advancing our understanding of associated pregnancy-related pathologies.

Synthetic GC, hydrocortisone, has been shown in the research literature to attenuate placenta nutrient uptake and decrease cell metabolism *in vivo* (310). Previous studies on JEG-3 cells, however, show that the more potent synthetic GC, dexamethasone, increases metabolic activity and proliferation in choriocarcinoma cell lines (311). Interestingly, dexamethasone decreases metabolic activity and proliferation in the BeWo choriocarcinoma cell line and it has been concluded that GCs may affect cell proliferation in a cell line-specific manner (311). Further, as the previously observed increase in JEG-3 cell proliferation after treatment with dexamethasone was not concentration-dependent, it is difficult to draw comparisons to the significantly less potent GC, hydrocortisone, where discrepancies in the cellular responses between the two remain largely unelucidated (312). The effect of GCs on SWAN-71 cell metabolic activity through the use of the MTT assay remains to be seen. Moreover, although previous research has shown that TGF-β1 increases cell metabolic activity and proliferation in JEG-3 cells, this also remains to be investigated in the SWAN-71 cell line (313). Finally, the effects of hydrocortisone and TGF-β1 in combination have not been investigated in any trophoblast cell model to date.

It was therefore the aim of the present study to investigate cell metabolic activity and cytotoxicity in SWAN-71 and JEG-3 cells using the MTT and lactate dehydrogenase (LDH) assays after treatment with hydrocortisone and TGF-β1, individually and in combination, to assess their suitability as trophoblast models for investigating the underlying mechanisms of stress- and inflammatory-related placental pathologies. Ultimately, by identifying appropriate models for future investigations, we move a step closer to advancing our understanding of the relationships between stress, inflammation, and trophoblast physiology which may lead to preventative or therapeutic measures for pregnancy-related pathologies such as PE, IUGR, and PTB.

2.2 Experimental aims and objectives

The present study aimed to investigate the effects of stress and inflammatory mediators, hydrocortisone and antagonist, mifepristone, as well as TGF-β1 and antagonist, SB431542, on trophoblast viability. The purpose of these experiments is to assess the suitability of pharmacologically-treated placental cell models for future investigations pertaining to the effects of stress and inflammatory mediators on trophoblast physiology to elucidate pathological mechanisms involved in the aetiologies of PE, IUGR, and PTB.

The following objectives were outlined to achieve this aim:

1. Determine the effects of 24-hour hydrocortisone, mifepristone, TGF-β1, SB431542, and respective vehicle treatments on SWAN-71 and JEG-3 cell metabolic activity using the MTT assay to provide an indication of trophoblast viability.
2. Determine the effects of 24-hour hydrocortisone, mifepristone, TGF-β1, SB431542, and respective vehicle treatments on SWAN-71 and JEG-3 cell cytotoxicity using the LDH assay to provide an indication of trophoblast viability.

2.3 Methods

2.3.1 Cell culture

SWAN-71 and JEG-3 human placental cell lines were kindly donated by Dr. Olivia Holland at passage 21 and 32, respectively, and cultured in Gibco™ Dulbecco's modified essential medium F-12 1:1 (DMEM/F12) (Thermo Fisher Scientific, Waltham, MA, USA) which was supplemented with 10% fetal bovine serum (FBS) and 1%

penicillin and streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) to make up complete growth media. Both cell lines were grown in T-75 flasks, incubated at 37 °C with 5% CO_2 and 95% O_2, and passaged as described by American Type Culture Collection (ATCC). Upon reaching approximately 90% confluency, growth medium was removed, cells were washed with Dulbecco's phosphate-buffered saline (DPBS), and then treated with 4 mL of 0.25%-EDTA trypsin (Thermo Fisher Scientific, Waltham, MA, USA). Cells were incubated at room temperature and observed under a light microscope until visibly detached from the culture flask. Following detachment, 6 mL of complete medium was added to neutralise trypsin activity. Cells were then dispensed into a 15 mL centrifuge tube and pelleted at 1000 RPM for five minutes at room temperature. Supernatant was discarded, cells were resuspended in 5 mL fresh medium, and then subcultured into a new T-75 flask at approximately 5 x 10^3 cells/cm^3. Cells numbers were determined using the haemocytometer counting method.

2.3.2 Pharmacological compounds

Hydrocortisone and mifepristone solutions were made up in ethanol to stock concentrations of 1 x 10^{-3} M. TGF-β1 was reconstituted in 5 mM sodium citrate to a stock concentration of 1 µg/mL. SB431542 was made up in dimethyl sulfoxide (DMSO) to form a stock concentration of 1 x 10^{-3} M. All pharmacological compounds were stored at -20 °C.

2.3.3 Cell viability assays

Cell viability assays were conducted to determine the metabolic and cytotoxic effects of the proposed pharmacological treatments and concentrations, as well as their respective vehicles, ethanol and DMSO. The MTT tetrazolium reduction assay and lactate dehydrogenase (LDH) assay were chosen to investigate metabolic activity and

cytotoxicity, respectively, providing complementary information about cell viability. Prior to pharmacologically-treated cell viability studies, both MTT and LDH assays were optimised with cell number titration and analysis at 3, 24, and 48 hours to determine the seeding density where cells exhibited exponential growth at 24 hours.

2.3.4 MTT assay

SWAN-71 and JEG-3 cells were subcultured in 96-well plates, seeded at 8,000 and 12,000 cells per well, respectively. Cells were incubated at 37 °C, 5% CO_2 and 95% O_2 for 24 hours to allow for adhesion. Thereafter, when cells were at least 50% confluent, the media was removed and pharmacologically-treated fresh media was added. The pharmacological treatments included hydrocortisone (100 nM, 200 nM, 500 nM, and 1 µM), mifepristone (1 nM, 5 nM, and 10 nM), a combination of hydrocortisone and mifepristone (100 nM + 10 nM, 200 nM + 10 nM, 500 nM + 10 nM, and 1 µM + 10 nM), ethanol vehicle control (0.01% w/v), TGF-B1 (5 ng/mL), SB413542 (1 µM), a combination of TGF-B1 and SB431542 (5 ng/mL + 1 µM), acidified saline vehicle control, DMSO (0.5 M, 0.01% w/v) vehicle control, as well as a combination of hydrocortisone and TGF-B1 (1 µM and 5 ng/mL). Furthermore, 10X lysis buffer (Promega, Madison WI, USA) was included as a control for maximal cell death. The cells were then incubated for 24 hours. Following the incubation period, treated media was discarded and wells were washed with DPBS, and the MTT assay was performed as previously described (314). MTT (50 µL) diluted in DPBS (1 mg/mL) was added to each well and cells were incubated for 4 hours to allow for the formation of purple formazan crystals which were solubilised in 100% DMSO. The plate was read at 570 nm on a Tecan Infinite 200 Pro Plate reader (Tecan Group Ltd, Mannedorf, Switzerland).

Viable cells metabolise MTT into formazan product which increases the absorbance reading, therefore it can be deduced that absorbance correlates to cell metabolic activity and provides an indication of cell viability (315).

2.3.5 LDH assay

SWAN-71 and JEG-3 cells were subcultured in 96-well plates, seeded at 8,000 and 12,000 cells per well, respectively. Cells were incubated at 37 °C, 5% CO_2 and 95% O_2 for 24 hours to allow for adhesion, then treated as previously described in the MTT assay. Following incubation, half of the treated media (50 µL) was removed from each well and dispensed into another 96-well plate with an equal volume of LDH solution (Promega, Madison, WI, USA) and incubated for 30 minutes. Next, 50 µL stop solution (Promega, Madison, WI, USA) was added to each well and the plate was read at 490 nm on a Tecan Infinite 200 Pro Plate reader (Tecan Group Ltd, Mannedorf, Switzerland).

The lactate, NAD^+, iodonitrotetrazolium violet (INT), and diaphorase contained in the LDH solution, when exposed to lactate dehydrogenase released from lysed cells, results in the reduction of INT to red-pigmented formazan, which increases the absorbance reading. It can therefore be deduced that absorbance correlates to cell lysis and provides an indication of cell death (316). The LDH assay, along with the information regarding cell metabolic activity obtained from the MTT assay, allows for a greater indication of overall cell viability.

2.3.6 Statistical analysis

Data for both the MTT and LDH assays were presented as mean ± standard deviation and analysed via one-way analysis of variance (ANOVA) in GraphPad Prism version 8 (California, USA) with Tukey's post-hoc multiple comparisons of means. A cut-off $p <$

0.05 denoted statistical significance. Both the MTT and LDH assays were carried out in three independent experiments (n = 3), each with triplicates.

2.4 Results

2.4.1 Effect of 24-hour drug treatment on SWAN-71 cells using the MTT assay

No significant differences were observed between vehicle and untreated control groups ($p > 0.05$) after 24 hours (see Figure 2.1). The SWAN-71 cell MTT assay showed a significant difference in cellular metabolism in all hydrocortisone treatment groups after 24-hour incubation when compared to untreated control cells (see Figure 2.1A). The hydrocortisone (500 nM) treatment elicited the greatest reduction in formazan production with -26.5% ($p = 0.0013$) compared to vehicle control, with the 200 and 100 nM treatment groups exhibiting -20.3% and -19.2% ($p = 0.0129$ and 0.0191, respectively). In contrast, hydrocortisone at a concentration of 1 µM had the lowest effect on SWAN-71 cell metabolism after 24 hours with -17.1%% compared to vehicle control ($p = 0.0416$). Mifepristone at concentrations of 5 and 1 nM decreased formazan production in SWAN-71 cells after 24 hours -30.7% and -29.4% ($p = 0.0081$ and $p = 0.0110$, respectively) (see Figure 2.1B). No significant difference was observed in the mifepristone (10 nM) treatment group compared to vehicle control ($p > 0.05$). Hydrocortisone treatments that were combined with mifepristone also resulted in significant decreases in formazan production in SWAN-71 cells (see Figure 2.1C). The combined hydrocortisone (1 µM) and mifepristone (10 nM) group exhibited -30.3% when compared to vehicle control ($p = 0.0002$). The other combined treatment group to cause a significant decrease in formazan production when compared to vehicle control was the hydrocortisone (200 nM) and mifepristone (10 nM), -18.4% ($p = 0.0189$).

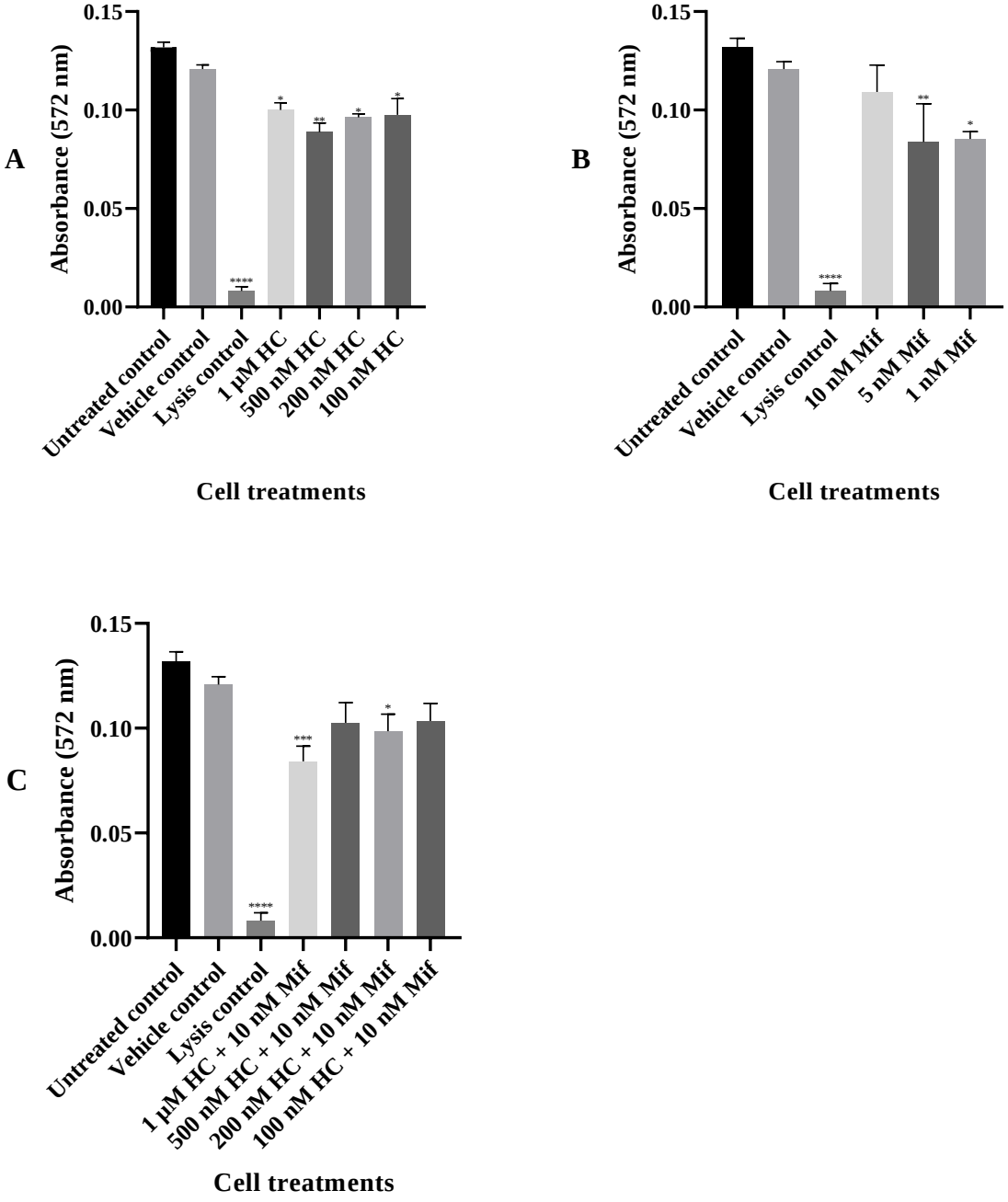


Figure 2.1. SWAN-71 cell metabolic activity after treatment with hydrocortisone and mifepristone for 24 hours utilising the MTT assay seeded at 8,000 cells/well. ***A.*** *Hydrocortisone (HC) with vehicle control;* ***B.*** *Mifepristone (Mif) with vehicle control;* ***C.*** *Hydrocortisone (HC) plus mifepristone (Mif) with vehicle control. Data presented as means ± SD, n = 3 for all treatment conditions. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared to vehicle control.*

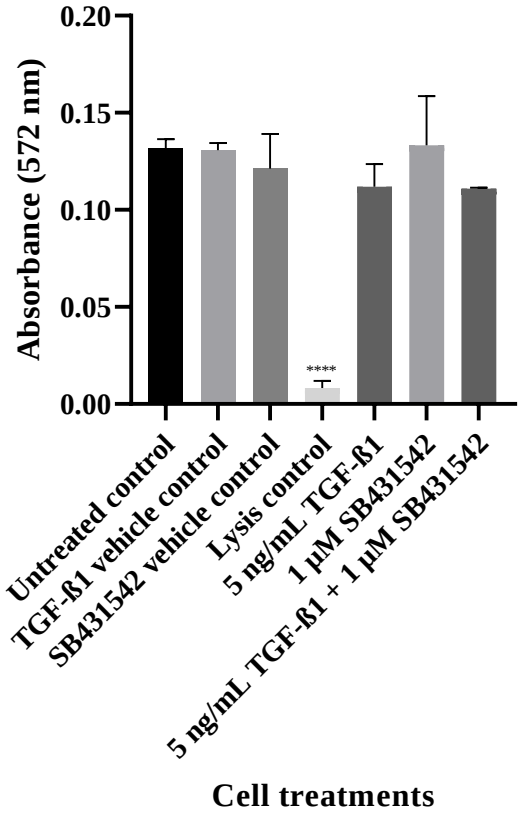


Figure 2.2. SWAN-71 cell metabolic activity after treatment with TGF-β1 and SB431542 for 24 hours utilising the MTT assay seeded at 8,000 cells/well. *TGF-β1, SB431542, TGF-β1 plus SB431542, and vehicle controls. Data presented as means ± SD, n = 3 for all treatment conditions. **** $p < 0.0001$ compared to vehicle control.*

The other two combined treatment groups of hydrocortisone (500 nM and 100 nM) and mifepristone (10 nM) did not cause significant changes in cell metabolism as measured by the MTT assay in SWAN-71 cells after 24 hours (-25.1% and -24.4%, respectively ($p > 0.05$)) (see Figure 2.1C). There were also no significant differences in formazan production between TGF-β1 (5 ng/mL) and SB431542 (1 µM) treatment groups and their respective vehicle controls after 24 hours incubation ($p < 0.05$) (see Figure 2.2).

2.4.2 Effect of 24-hour drug treatment on JEG-3 cells using the MTT assay

No significant differences were observed between untreated and vehicle controls ($p > 0.05$) after 24 hours treatment in JEG-3 cells (see Figure 2.3). The MTT assay showed significant differences in cell metabolism in the hydrocortisone treatment groups compared to vehicle control, 1 μM (-17.3%, $p = 0.0286$), 200 nM (-18%, $p = 0.0224$), and 100 nM (-17%, $p = 0.0322$), whereas there was no significant difference in the hydrocortisone 500 nM treatment group ($p > 0.05$) (see Figure 2.3A). For the mifepristone treatment groups, 24 hours treatment with 10 or 5 nM were significantly different to vehicle control for the JEG-3 cells (-34.5%, $p = 0.0010$ and -35.0%, $p = 0.0009$, respectively) (see Figure 2.3B). No statistical differences were observed when hydrocortisone combined with 10 nM mifepristone treatment groups compared to vehicle control. After 24 hours, TGF-β1 (5 ng/mL), SB431542 (1 μM), as well as combined TGF-β1 and SB431542 treatment groups exhibited no statistically significant differences to respective vehicle controls for the JEG-3 cells (see Figure 2.4).

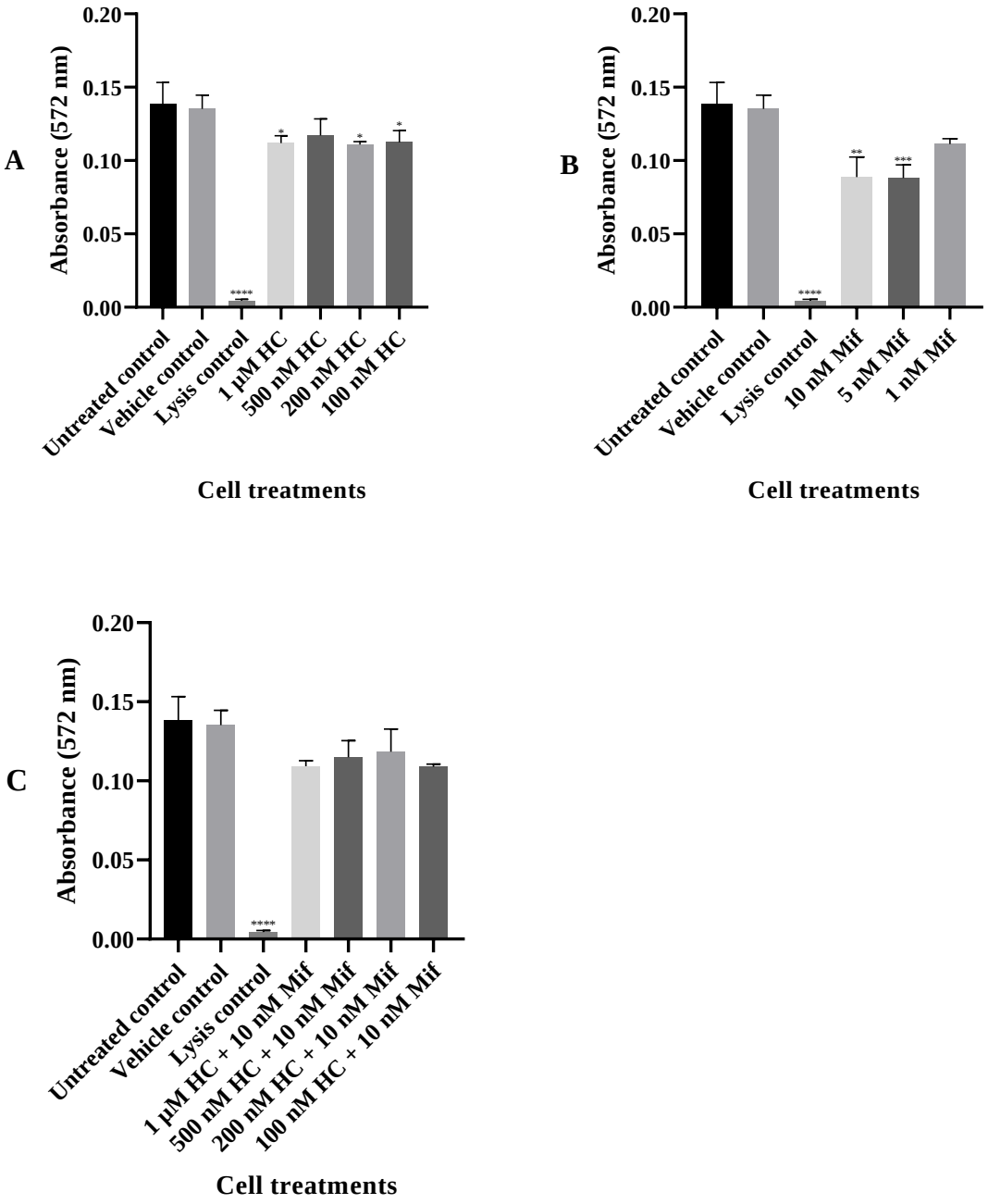


Figure 2.3. JEG-3 cell metabolic activity after treatment with hydrocortisone and mifepristone for 24 hours utilising the MTT assay seeded at 12,000 cells/well.

A. *Hydrocortisone (HC) with vehicle control;* ***B.*** *Mifepristone (Mif) with vehicle control;* ***C.*** *Hydrocortisone (HC) plus mifepristone (Mif) with vehicle control. Data is presented as means ± SD, n = 3 for all treatment conditions. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared to vehicle control.*

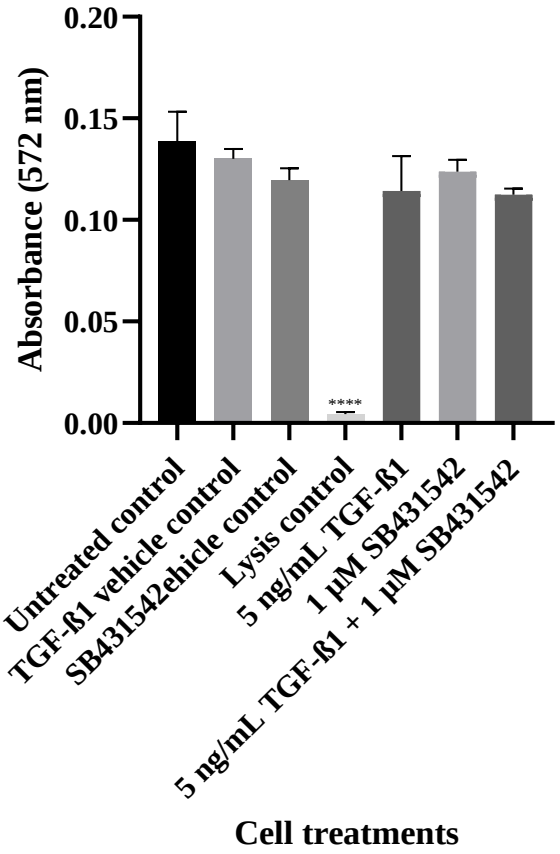


Figure 2.4. JEG-3 cell metabolic activity after treatment with TGF-β1 and SB431542 for 24 hours utilising the MTT assay seeded at 12,000 cells/well. *TGF-β1, SB431542, TGF-β1 plus SB431542, and vehicle controls. Data is presented as means ± SD, n = 3 for all treatment conditions. **** $p < 0.0001$ compared to vehicle control.*

2.4.3 Effect of 24-hour drug treatment on SWAN-71 cells using the LDH assay

In the SWAN-71 cells, there were no statistically significant differences between the vehicle control and the untreated control groups ($p > 0.05$) following 24 hours incubation, indicating that the vehicle controls (ethanol, DMSO, and sodium citrate) did not cause cell death (see Figure 2.5). As expected, a significant increase in cell death was observed with the lysis control when compared to vehicle and untreated control SWAN-71 cells groups ($p > 0.05$).

With respect to 24-hour drug treatment with hydrocortisone, mifepristone, or combined treatment, there were no statistically significant differences between the pharmacological treatment groups and their respective vehicle controls in the SWAN-71 cell LDH cytotoxicity assays ($p > 0.05$) (see Figures 2.5 and 2.6).

2.4.4 Effect of 24-hour drug treatment on JEG-3 cells using the LDH assay

Similarly, for the JEG-3 cell LDH cytotoxicity assay, there were no significant differences between the pharmacological treatment groups of TGF-β1 (5 ng/mL) or TGF-β1 antagonist SB431542 (1 μM) and their respective vehicle controls, nor between vehicle and untreated control groups after 24-hour incubation ($p > 0.05$) (see Figures 2.7 and 2.8).

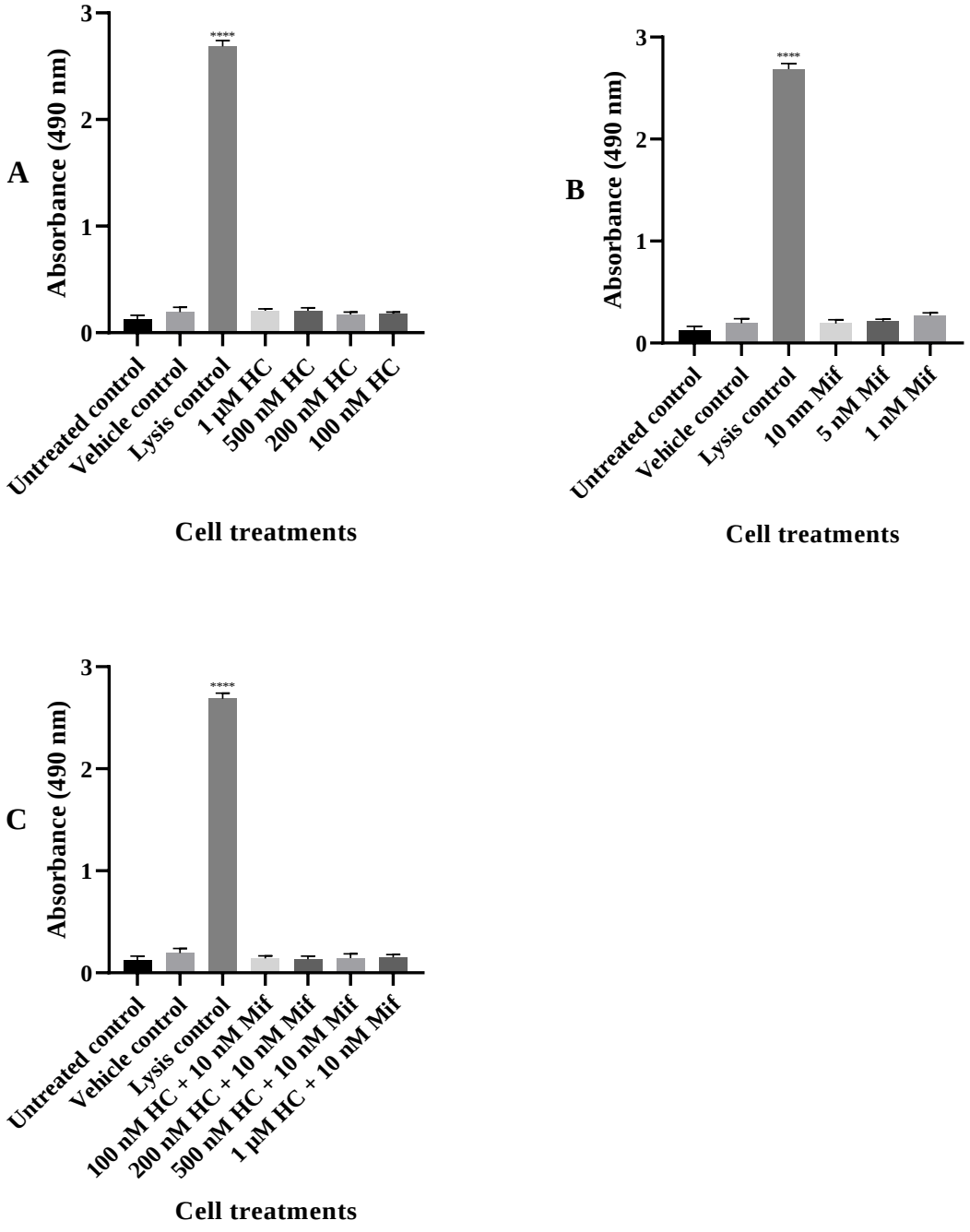


Figure 2.5. SWAN-71 cell cytotoxicity after treatment with hydrocortisone and mifepristone for 24 hours utilising the LDH assay seeded at 8,000 cells/well.

A. *Hydrocortisone (HC) with vehicle control;* ***B.*** *Mifepristone (Mif) with vehicle control;* ***C.*** *Hydrocortisone (HC) plus mifepristone (Mif) with vehicle control. Data is presented as means ± SD, n = 3 for all treatment conditions. **** $p < 0.0001$ compared to vehicle control.*

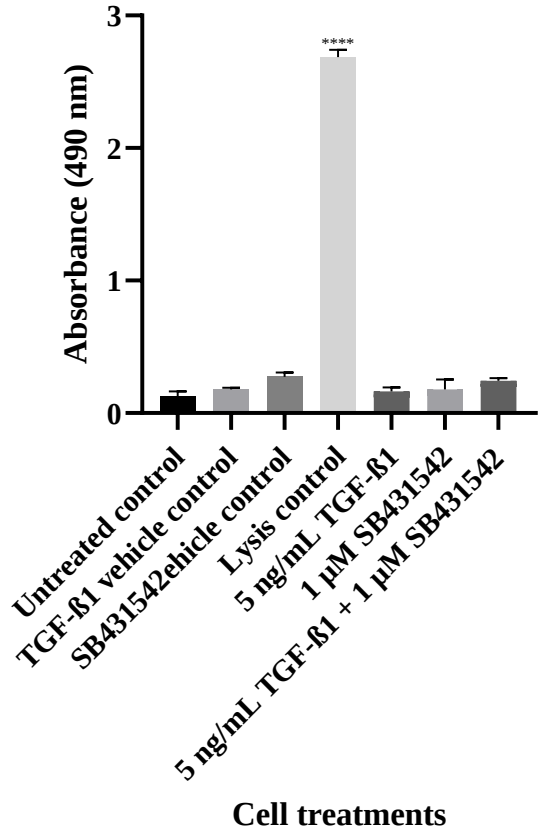


Figure 2.6. SWAN-71 cell cytotoxicity after treatment with TGF-β1 and SB431542 for 24 hours utilising the LDH assay seeded at 8,000 cells/well. *TGF-β1, SB431542, TGF-β1 plus SB431542, and vehicle controls. Data is presented as means ± SD, n = 3 for all treatment conditions. **** p < 0.0001 compared to vehicle control.*

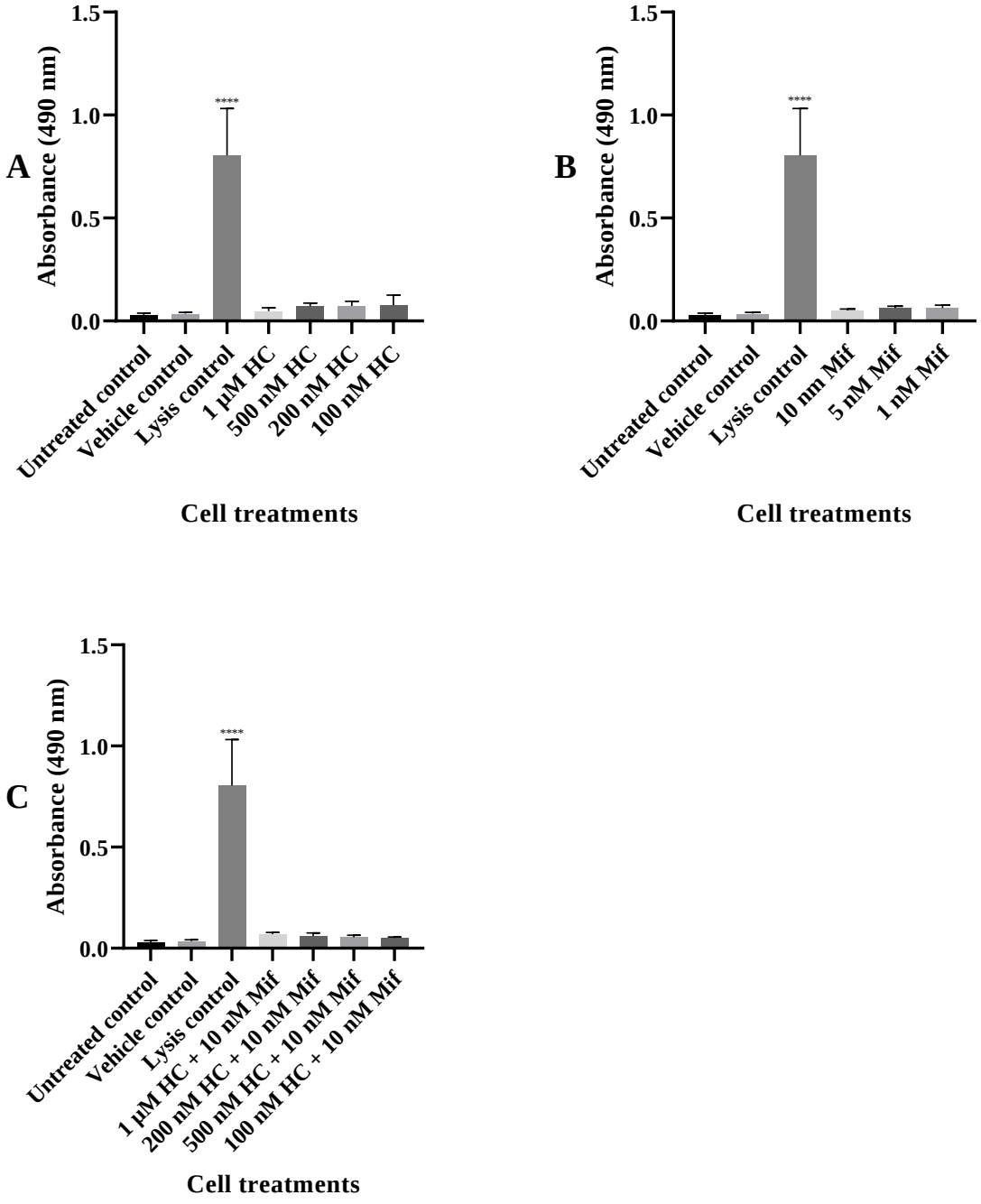


Figure 2.7. JEG-3 cell cytotoxicity after treatment with hydrocortisone and mifepristone for 24 hours utilising the LDH assay seeded at 12,000 cells/well. *A.* *Hydrocortisone (HC) with vehicle control;* ***B.*** *Mifepristone (Mif) with vehicle control;* ***C.*** *Hydrocortisone (HC) plus mifepristone (Mif) with vehicle control. Data is presented as means ± SD, n = 3 for all treatment conditions. **** $p < 0.0001$ compared to vehicle control.*

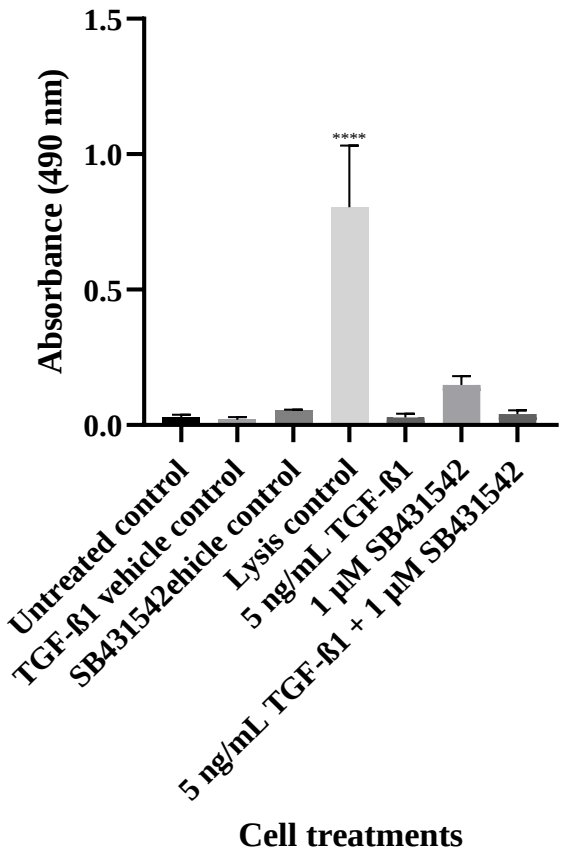


Figure 2.8. JEG-3 cell cytotoxicity after treatment with TGF-β1 and SB431542 for 24 hours utilising the LDH assay seeded at 12,000 cells/well. *TGF-β1, SB431542, TGF-β1 plus SB431542, and vehicle controls. Data is presented as means ± SD, n = 3 for all treatment conditions. *** $p < 0.001$ compared to vehicle control.*

2.5 Discussion

To ascertain the effects of high concentrations of hydrocortisone and TGF-β1 on the viability of the placental cell models, SWAN-71 and JEG-3, MTT and LDH assays were carried out over a period of 24 hours.

Firstly, there were no significant differences in metabolic activity between each of the vehicle controls and untreated controls, which indicated that the chosen vehicles did not affect cell viability with respect to metabolism to a statistically significant extent and, therefore, allowing for reliable assessment of the pharmacological compounds. Next, cell lysis treatment groups served as negative controls and exhibited the greatest decrease in cell metabolic activity as expected. With respect to the pharmacological compounds, a significant reduction in cell metabolic activity was observed for all hydrocortisone treatments relative to vehicle controls in SWAN-71 cells, as well as hydrocortisone (100, 200 nM, and 1 μM) treatment groups in JEG-3 cells. These findings were consistent with previous reports that excess hydrocortisone attenuates placenta nutrient uptake and decreases cell metabolism (310).

Contrastingly, JEG-3 cells exhibited increased metabolic activity and proliferation in a study investigating the effects of dexamethasone on choriocarcinoma cell lines (311). The same experiment, however, also observed decreased metabolic activity and proliferation in BeWo choriocarcinoma cells and concluded that glucocorticoids may affect cell proliferation in a cell line-specific manner (311). Further, as the increased JEG-3 cell proliferation after dexamethasone treatment was not concentration-dependent, it was difficult to draw comparisons to the significantly less potent hydrocortisone used in the present study especially as discrepancies in the cellular responses between the two glucocorticoids remains largely unelucidated (312).

Mixed results were observed in the mifepristone treatment groups with the 1 and 5 nM treated SWAN-71 cells and the 5 and 10 nM treated JEG-3 cells exhibiting decreased cell metabolism while mifepristone at 10 nM in SWAN-71 cells and mifepristone at 1 nM in JEG-3 cells had no significant effect. Mifepristone has been demonstrated to exert potent anti-proliferative effects in the trophoblast cell line, HTR-8/SVneo, as well as in endometrial and ovarian carcinoma cell lines which corroborated the findings of the present study (317-319). Interestingly, in the mifepristone (10 nM) treatment group for SWAN-71 cells, cell metabolism was not significantly affected.

Moreover, significant reductions in cell metabolism were also observed in the 200 nM and 1 µM hydrocortisone treatments combined with mifepristone (10 nM) in SWAN-71 cells while none of the JEG-3 groups were affected. This is an interesting result as neither hydrocortisone nor mifepristone affected SWAN-71 or JEG-3 cell metabolic activity in a concentration-dependent manner. Further, the combined hydrocortisone and mifepristone treatments failed to exhibit synergetic or inhibitory effects in both the 100 and 500 nM treatment groups for SWAN-71 cells and in all treatment groups for JEG-3 cells. These observations may be due to the glucocorticoid agonist properties of mifepristone which have been seen in particular cell types and growth conditions, where it has been demonstrated that such agonist activity was independent of changes of dose-response relationships (320). In this case, the specific effects of mifepristone on formazan production in metabolically active cells warrants further investigation. Additionally, this observation could also be a source of error within the present study.

There were no statistically significant changes in cell metabolism in the TGF-β1 (5 ng/mL), SB431542 (1 µM), and combined treatment groups for SWAN-71 nor JEG-3 cells after 24 hours. These findings were in contrast with a previous study investigating the effect of TGF-β1 (5 ng/mL) on JEG-3 cell metabolism through use of the MTT

assay which demonstrated increased cell metabolism and proliferation after 6 hours of treatment, at the same concentration investigated in the present study (313).

It is important to note that the MTT assay only provides information regarding cellular metabolic activity and is an inadequate independent measure of overall cell survival. However, when utilised in concert with the LDH cytotoxicity assay, a more reliable indication of absolute cell viability follows. In each of the LDH assays, there were no significant differences between the vehicle and untreated controls which allowed for adequate assessment of the cytotoxicity of the pharmacological compounds of the present study. Interestingly, for both cell lines, SWAN-71 and JEG-3, there were no statistically significant differences in cytotoxicity between each of the pharmacological compounds and their respective vehicle controls. These results corroborated previous findings that dexamethasone (100 nM) had no effect on SWAN-71 cell cytotoxicity (151). Cell lysis groups were used as positive controls for the LDH assay which elicited the greatest amount of cell death and indicated that the assay was functional.

Taken together, the MTT and LDH assays demonstrated that although the pharmacological compounds were indeed interfering with cellular metabolism and proliferation to a measurable extent, they were not causing cell death. In summary, the findings of the MTT and LDH assays confirmed that both hydrocortisone and TGF-β1 at the selected concentrations seldom affected total cell viability and both SWAN-71 and JEG-3 cell lines could subsequently be utilised as *in vitro* models for investigating the relationships between stress, inflammation, and trophoblast physiology.

CHAPTER 3: GR mRNA and protein expression and SERT protein expression in SWAN-71 and JEG-3 cells after treatment with hydrocortisone or TGF-β1 for 24 hours

3.1 Introduction

Gestational pathologies such as PE and IUGR affect up to 10 and 15% of pregnancies in the developed world (1, 4), respectively, and can lead to devastating fetal outcomes such as PTB which affects 15 million pregnancies each year (12). Recently, perturbations in stress and inflammatory mediators, cortisol and TGF-β1, have been implicated in these pathological conditions as well as maternal complications, GDM and PD (19, 140-144, 157-159, 162, 201, 209-217). In what is considered a normal physiological phenomenon, maternal cortisol and TGF-β1 levels have been shown to increase throughout gestation in a carefully controlled manner (96, 200, 201). Cortisol has important roles in normal fetal development and prepares the conceptus for extrauterine existence (85, 94, 97, 98, 145). When normal cortisol physiology is disrupted, such as in cases of maternal stress (114), malnourishment (115, 116), depression (166), obesity (118), GC medications (119), as well as the saturation or downregulation of 11β-HSD (120, 121, 145), excess cortisol may interfere with normal trophoblast function at the maternofetal interface and contribute to pregnancy-related pathologies. Similarly, various inflammatory cytokines such as TGF-β1 are differentially expressed throughout the course of pregnancy. TGF-β1 has been shown in the research literature to be upregulated during pregnancy with crucial roles in fetal development (200, 201). TGF-β1 is a potent regulator of fibrosis and excess levels have been associated with various pregnancy-related pathologies, such as PE and PTB, with reports of interfering with trophoblast invasive activity (201-205, 209). Perturbations in

cortisol and TGF-β1 signalling during early gestation, particularly in cases of excess concentrations, appear to play a significant role in pregnancy-related complications that result from disrupted trophoblast function. Indeed, cortisol and TGF-β1 are extremely complex and context-dependent endocrinological systems and their interactions with the GR and SERT may play key roles in the aetiology of pregnancy-related pathologies. Excess levels of cortisol and TGF-β1 have been shown to affect the expression of GR variant isotypes and SERT protein in various cell types. For instance, excess cortisol observed in pre-term placentae has been demonstrated to upregulate GRβ, GRα-C, and GRα-D variants (122-124). Further, excess glucocorticoids have been reported to down regulate GRα-A and upregulate GRβ in various cell types including human monocyte THP-1 cell line and peripheral blood mononuclear leukocytes (130, 321, 322). TGF-β1, on the other hand, reduces GRα-A expression in the lung adenocarcinoma A549 cell line, modulates GC sensitivity through interactions with GR-β in epithelial cells, and also silences GR transcriptional activity in rat liver *in vivo* (303). Contrasting results have been seen in U-937 monocyte cell line studies with TGF-β1 upregulating both GR mRNA and protein expression in a dose-dependent manner as well as optimising the binding of GCs (305). TGF-β1 interactions with the GR have seldom been investigated in placental models and warrants further research. With respect to the SERT protein, excess cortisol has been shown to increase its expression in both human and rodent studies although the underlying mechanisms remain unknown (269-272). TGF-β1 has also been demonstrated to increase SERT expression and enhance its function in intestinal cell studies (295, 296).

In a novel attempt to investigate these interactions in placental cell models, and to corroborate findings of changes in GR protein expression due to increased GCs, the present study aimed to measure GR isotype and SERT protein expression in JEG-3 and

SWAN-71 cell lines after treatment with supraphysiological concentrations of hydrocortisone and TGF-β1. SWAN-71 and JEG-3 have both been cited extensively in the literature as adequate *in vitro* models of placental physiology. Additionally, while alterations in protein expression are significant, it seldom explains the underlying mechanisms at play and says very little about genetic expression. Recent reviews have remarked that although correlations between mRNA and protein expression levels might be useful, they are inadequate when it comes to predicting corresponding expression (323-325). This also highlights the impact of post-transcriptional, translational and degradation regulation in the determination of protein expression levels (323). It was therefore the aim of the present study to investigate GR mRNA expression in SWAN-71 cells to advance our understanding of the relationships between stress, inflammatory, and serotonin system processes in trophoblast pathophysiology with respect to gene expression. Ultimately, by narrowing the gap in our understanding of the relationship between stress, inflammation, and serotonin mechanisms in early gestation, we move a step closer to potential therapeutic or preventative measures for associated pregnancy-related complications.

3.2 Experimental aims and objectives

The present study aimed to investigate the effects of stress and inflammatory mediators, hydrocortisone and antagonist, mifepristone, as well as TGF-β1 and antagonist, SB431542 on GR isotype and SERT protein expression as well as total GR mRNA expression to elucidate their potential roles in human trophoblast pathophysiology. The purpose of these experiments was to elucidate the effects of stress and inflammatory mediators on trophoblast physiology to determine the pathological mechanisms involved in the aetiologies of PE, IUGR, and PTB.

The following objectives were outlined to achieve this aim:

1. Determine changes in protein expression of GR isotypes and the SERT following 24-hour treatments with hydrocortisone, mifepristone, TGF-β1, SB431542, and respective vehicles using Western blot analysis.
2. Investigate changes in total GR mRNA expression following 24-hour treatments with hydrocortisone, mifepristone, TGF-β1, SB431542, and respective vehicles in SWAN-71 cells using NanoString nCounter analysis.

3.3 Methods

3.3.1 Cell culture

SWAN-71 and JEG-3 cells were cultured as previously described in Chapter 2.3.1.

3.3.2 Pharmacological compounds

Drug treatments were carried out as previously described in Chapter 2.3.2. Cell viability results ultimately allowed for key decisions to be made regarding the concentrations of hydrocortisone and mifepristone to be used for subsequent experiments (see Chapter 2). Hydrocortisone, at a concentration of 500 nM, was chosen as the physiological reference of maternal circulating cortisol as it is a good baseline representation of blood plasma cortisol levels during gestation and did not exhibit cytotoxic effects (109, 131-136). For comparison, given that the literature has indicated that cortisol levels can reach 1 µM during gestation and as this treatment group was also not cytotoxic, it was selected as the high cortisol concentration in subsequent experiments (109). Moreover, mifepristone at a concentration of 10 nM was selected as it similarly was not cytotoxic and has been shown in literature to have more than three times the binding affinity for the GR than dexamethasone and reduced its binding by up to 75% (326, 327). As

dexamethasone is a more potent glucocorticoid than hydrocortisone, mifepristone (10 nM) is a suitable antagonist of the GR in the present study.

3.3.3 Pharmacological treatments of cells for Western blot analysis

SWAN-71 and JEG-3 cells were subcultured into 6-well plates, seeded at approximately 150,000 cells per well. Cells were incubated at 37 °C, 5% CO_2 and 95% O_2 for 24 hours to allow for adhesion. When cells were more than 50% confluent, the media was removed, and pharmacologically-treated fresh media was added. Pharmacological treatments included hydrocortisone (500 nM and 1 µM), mifepristone (10 nM), a combination of hydrocortisone and mifepristone (1 µM + 10 nM, respectively), ethanol vehicle control (0.01% w/v), TGF-β1 (5 ng/mL), SB413542 (1 µM), a combination of TGF-B1 and SB431542 (5 ng/mL and 1 µM, respectively), and TGF-β1 vehicle control (5 x 10^{-3} mM sodium citrate), and DMSO vehicle control (0.5 M, 0.01% w/v). Cells were incubated for 24 hours. Pharmacological treatments were conducted in triplicates.

3.3.4 Protein extraction for Western blot analysis

Following 24-hour pharmacological treatment, the media was discarded, and cells were washed with ice-cold DPBS then subsequently removed via cell scraping. DPBS cell suspensions from each respective well were then decanted into a microtube and centrifuged into a pellet at 1000 RPM for five minutes at 4 °C. Cells were then resuspended in 2X RIPA lysis buffer (Merrick Millipore, Burlington, MA, USA) supplemented with protease and phosphatase inhibitors (Biotool LLC, Houston, TX, USA) and mechanically lysed through vigorous aspiration with a pipette tip. Lysates were then left to incubate on ice for 30 minutes before being centrifuged at 12000 RPM for 15 minutes at 4 °C. Protein-containing supernatants were collected and transferred to new microtubes for protein quantity estimation.

3.3.5 BCA assay for Western blot analysis

Whole-cell lysate protein quantities were estimated using the bicinchoninic acid (BCA) assay. Conservative samples of each cell lysate were plotted against a standard curve of known concentrations of bovine serum albumin (BSA). This was done by adding the provided BCA kit reagents (Thermo Fisher Scientific, Waltham, MA, USA) as per manufacturer's instructions on a 96-well plate read at 542 nm on a Tecan Infinite 200 Pro plate reader (Tecan Group Ltd, Mannedorf, Switzerland). Cell lysates were immediately stored at -80 °C prior to subsequent aliquoting and Western blot analysis.

3.3.6 Western blot analysis

Western blot analysis was carried out to measure the protein expression between the cell treatment groups in a semi-quantitative manner. Lysates were thawed on ice before adding 4X Laemmli loading buffer, containing β-mercaptoethanol, in a 1:3 ratio. Samples were heated at 95 °C for five minutes and loaded into 10% precast polyacrylamide TGX gels (Bio-Rad, Hercules, CA, USA) at 40 µg per well. Pageruler™ Plus Prestained Ladder (Thermo Fisher Scientific, Waltham, MA, USA) was utilised as the protein ladder to visualise and identify protein molecular weights. Electrophoresis was carried out at 100 V for approximately 90 minutes. Gels were then transferred to polyvinylidene difluoride (PVDF) membranes supplied in Trans-Blot® Turbo™ Mini PVDF transfer packs via the Trans-Blot® Turbo™ Transfer System (Bio-Rad, Hercules, CA, USA). Following transfer, the membranes were washed for 10 minutes with tris-buffered saline (TBS) then incubated with Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE, USA) for one hour. After decanting blocking buffer, the membranes were incubated for 16 hours with primary antibodies at 4 °C in the dark with agitation. SERT proteins were detected by 1:250 polyclonal Goat Anti-SLC6A4

antibody (Thermo Fisher Scientific, Waltham, MA, USA) with immunogen binding of synthetic peptide sequence (LHQGERETWGK) corresponding to the internal amino acids of SCL6A4 while GR proteins were detected by 1:1000 polyclonal Rabbit Anti-NR3C1 (Bethyl Laboratories, Montgomery, TX, USA) with immunogen binding between 150 and 200. 1: 30,000 Mouse Anti-Cofilin (Cell Signalling Technology Inc., Danvers, MA, USA) was used to detect cofilin housekeeping proteins as a loading control measure. Primary antibodies were recycled, and membranes were washed 4 times with TBS with tween (TBST) for five minutes before a final wash with TBS. Membranes were then incubated with secondary antibodies, 1:15,000 Donkey Anti-Goat, 1:30,000 Goat Anti-Rabbit, or 1:500 Goat Anti-Mouse (Li-Cor Biosciences, Lincoln, NE, USA), for one hour at room temperature in the dark with agitation. Secondary antibodies were similarly recycled, and membranes were washed once more with TBST for five minutes, 4 times, followed by a final wash with TBS for 10 minutes. Thereafter, membranes were dried and then visualised using an Odyssey CLx Imaging System (Li-Cor Biosciences, Lincoln, NE, USA). Subsequent analysis included normalisation via cofilin loading control, as well as internal standard pooled A549 cell line lysate sample as positive control between all gels. Band intensities were quantitated using ImageStudio (Li-Cor Biosciences, Lincoln, NE, USA). Western blot analyses were conducted in technical duplicates for each of the pharmacological treatment triplicates.

3.3.7 Probe design for NanoString nCounter analysis

The NanoString nCounter analysis system allows for the highly sensitive and quantitative assessment of gene expression at the cellular level (327). The RNA sequence for the GR, *NR3C1*, was submitted to NanoString Bioinformatics (NanoString, Seattle, WA) for the design of the CodeSet. The CodeSet comprises

target-specific probes each with a capture probe, a reporter probe, and a protector probe. A unique barcode of fluorescent signals known as a reporter tag is bound to the 3' end of each reporter probe while a universal biotin tag binds the 5' end of each capture probe. During hybridisation, both probes complex with specific continuous sequences on target RNA where the fluorescent reporter tags are then imaged by a digital analyser and subsequently counted.

3.3.8 Pharmacological treatments of cells for NanoString nCounter analysis

SWAN-71 were subcultured into 12-well plates, seeded at approximately 75,000 cells per well. Cells were incubated at 37 °C, 5% CO_2 and 95% O_2 for 24 hours to allow for adhesion. When cells were more than 50% confluent, the media was removed, and pharmacologically-treated fresh media was added. Pharmacological treatments included hydrocortisone (1 µM), ethanol vehicle control (0.01% w/v), TGF-β1 (5 ng/mL), SB413542 (1 µM), and DMSO vehicle control (0.5 M, 0.01% w/v). Cells were incubated for 24 hours. Pharmacological treatments were conducted in triplicates.

3.3.9 Nucleic acid extraction for NanoString nCounter analysis

Following 24-hour pharmacological treatment, media was discarded, cells were washed with ribonuclease-free water (QIAGEN, Venlo, NL), and subsequently removed via cell scraping. Cell suspensions from each respective well were then decanted into a microtube and centrifuged into a pellet at 1000 RPM for five minutes at 4 °C. Supernatant was discarded before cells were then resuspended in RLT buffer (QIAGEN, Venlo, NL) supplemented with β-mercaptoethanol and mechanically lysed through vortex mixing for one minute. Nucleic acid-containing lysates were then stored at -80 °C.

3.3.10 RNA titration for NanoString nCounter analysis

To optimise the sample input and avoid signal saturation and experimental failure, nucleic acid samples were titrated using the PlexSet Titration Kit (NanoString Technologies, Seattle, WA). nCounter XT TagSet was thawed to room temperature, mixed, and gently spun down on a picofuge. 1, 3, or 5 µL of sample were added to 12 tubes of a master mix containing the nCounter XT TagSet, probes containing TE-Tween (10 mM Tris pH 8, 1 mM EDTA, 0.1% Tween-20), and hybridisation buffer (NanoString Technologies, Seattle, WA). Samples were incubated in a thermocycler for 16 hours at 67 °C. The sample-containing 12-tube strip was loaded on to a cartridge and subsequently scanned on a digital analyser using a custom Titration Kit RLF from the NanoString Bioinformatics team (NanoString Technologies, Seattle, WA). The data were analysed with nSolver software (NanoString Technologies Seattle, WA) to determine the optimal sample input for the PlexSet experiment.

3.3.11 NanoString nCounter analysis

The NanoString nCounter protocol was carried out as per manufacturer's instructions (NanoString Technologies, Seattle, WA). PlexSet assay hybridization components, hybridization buffer, PlexSet reagents, Probes A and B, nuclease-free water, were collected and pipetted into each well of a 96-well plate. The plate was incubated overnight at 67 °C for hybridization avoiding evaporation. Experimental samples were then added to each well and mixed via pipette. The plate was then incubated in a thermocycler for 24 hours at 67 °C and then ramped down to 4 °C. Thereafter, the plate was spun at 2,000 g and allowed to stop. The samples were pooled from the plate, by columns, into a strip tube which was then loaded onto the workstation for detection. The custom RLF from the NanoString Bioinformatics team (NanoString Technologies,

Seattle, WA) was loaded onto the digital analyser and the cartridge was scanned. mRNA counts were normalized to the housekeeping genes, GUSB, PGK1, and ABCF1, with nSolver software (NanoString Technologies Seattle, WA).

3.3.12 Statistical analysis

Western blot data were analysed via one-way ANOVA in GraphPad Prism version 8 (California, USA) with Tukey's post-hoc multiple comparisons of means. NanoString nCounter analysis data were analysed via unpaired student T-test with Welch's correction All data were presented as mean ± standard deviation with a cut-off $p < 0.05$ for statistical significance. All pharmacological treatments were carried out in triplicates ($n = 3$) and Western blots were carried out in technical duplicates.

3.4 Results

3.4.1 Effect of 24-hour drug treatment on GR protein expression in SWAN-71 cells using Western blot analysis

The total GR antibody (Bethyl Laboratories, Montgomery, TX, USA) detected approximately 12 specific bands in whole SWAN-71 cell lysates (n = 3) after the SWAN-71 cells were treated with hydrocortisone (500 nM or 1 µM), mifepristone (10 nM), TGF-β1 (5 ng/mL), or SB431542 (1 µM) for 24 hours. Among these bands, molecular weights of 94, 91, 81, 74, 74, 65, and a cluster of blots between 50-55 kDa were observed that are consistent with known isoforms GRα-A (94 kDa), GRβ (91 kDa), GRα-C (81 kDa), GRP (74 kDa), GRA (65 kDa), and GRα-D1-3 (50-55 kDa). Several unknown bands at molecular weights of 250, 130, 45, and 38 kDa were also observed.

The highest hydrocortisone concentration (1 µM) did not significantly alter GRβ, GRα-C, GRP, GRA, and GRα-D compared to vehicle control (0.01% ethanol w/v) ($p > 0.05$). However, 24-hour treatment with hydrocortisone (1 µM) caused a significant decrease in the GRα-A isotype protein expression compared to vehicle control (-32%, $p = 0.0008$) (see Figures 3.1 and 3.2). No significant alterations in GR isotype expression were seen following 24-hour incubations with mifepristone (10 nM), hydrocortisone (500 nM), nor hydrocortisone and mifepristone combined treatment groups in SWAN-71 cells ($p > 0.05$). These results show that the GR receptor antagonist mifepristone reversed the effect of hydrocortisone (1 µM) following 24 hours incubation in SWAN-71 cells.

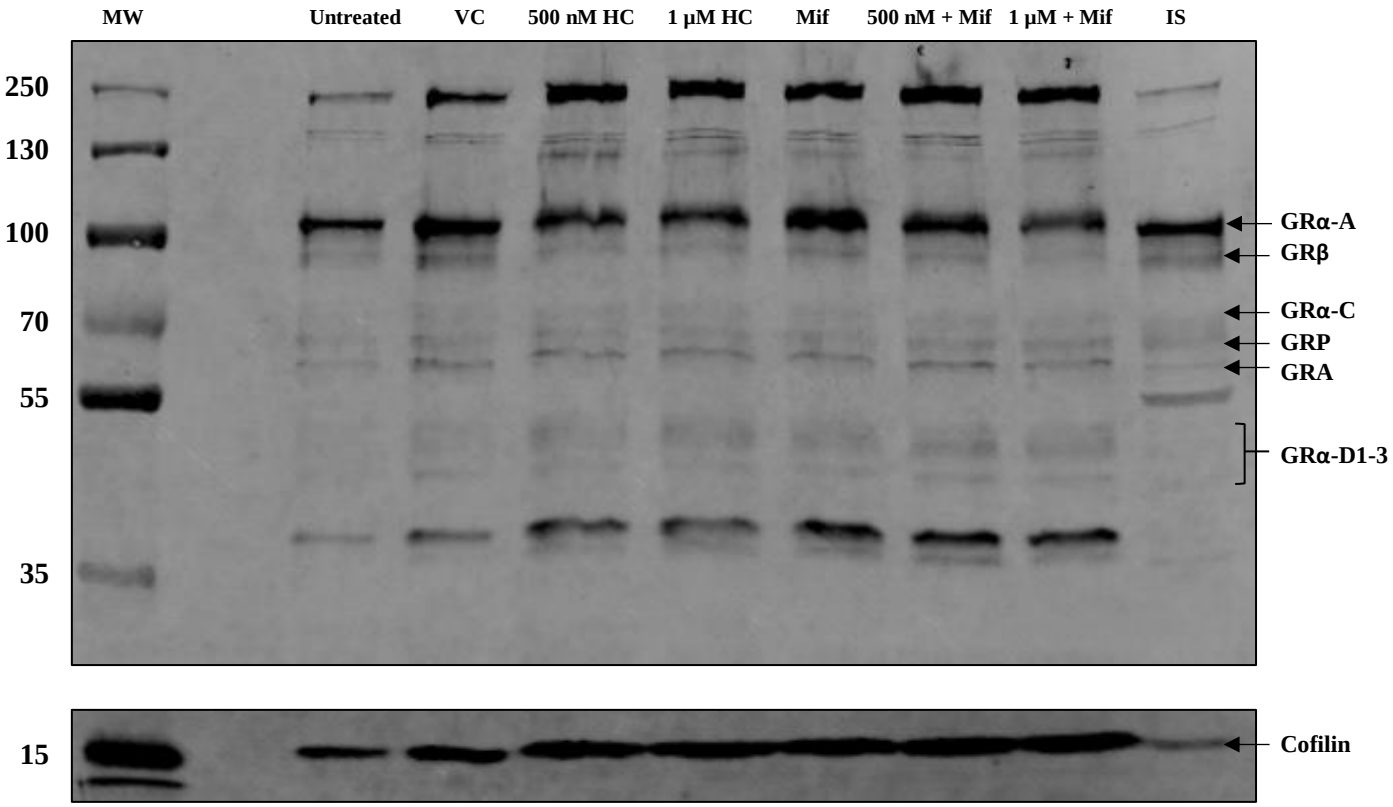
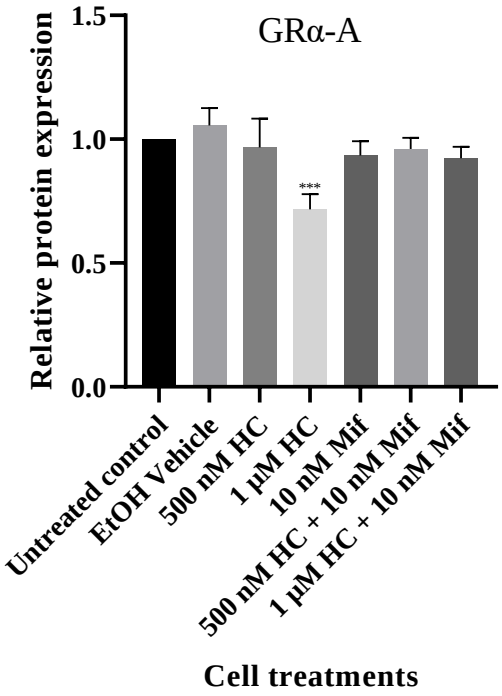
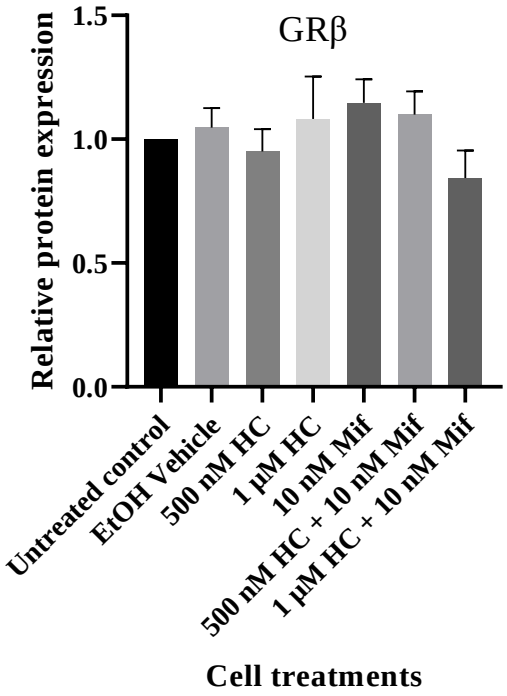
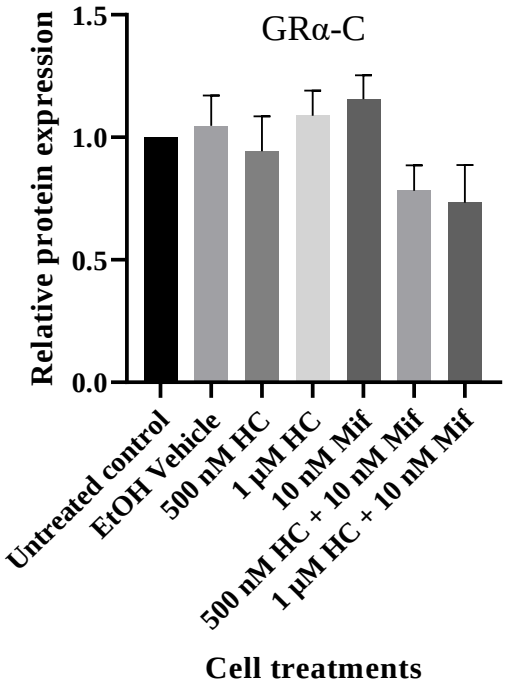


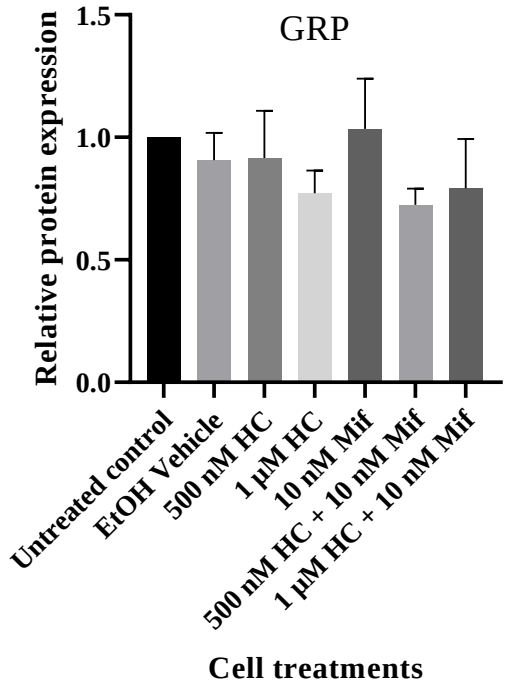
Figure 3.1. **Representative Western blot of GR protein expression in SWAN-71 cells after 24-hour treatment with hydrocortisone and mifepristone.**

MW, molecular weight (kDa); VC, vehicle control; HC, hydrocortisone; Mif, mifepristone (10 nM); IS, internal standard (A549 cell lysate).


GRα-A
Relative protein expression
1.5
1.0
0.5
0.0
***
Untreated control
EtOH Vehicle
500 nM HC
1 μM HC
10 nM Mif
500 nM HC + 10 nM Mif
1 μM HC + 10 nM Mif
Cell treatments


GRβ
Relative protein expression
1.5
1.0
0.5
0.0
Untreated control
EtOH Vehicle
500 nM HC
1 μM HC
10 nM Mif
500 nM HC + 10 nM Mif
1 μM HC + 10 nM Mif
Cell treatments


GRα-C
Relative protein expression
1.5
1.0
0.5
0.0
Untreated control
EtOH Vehicle
500 nM HC
1 μM HC
10 nM Mif
500 nM HC + 10 nM Mif
1 μM HC + 10 nM Mif
Cell treatments


GRP
Relative protein expression
1.5
1.0
0.5
0.0
Untreated control
EtOH Vehicle
500 nM HC
1 μM HC
10 nM Mif
500 nM HC + 10 nM Mif
1 μM HC + 10 nM Mif
Cell treatments

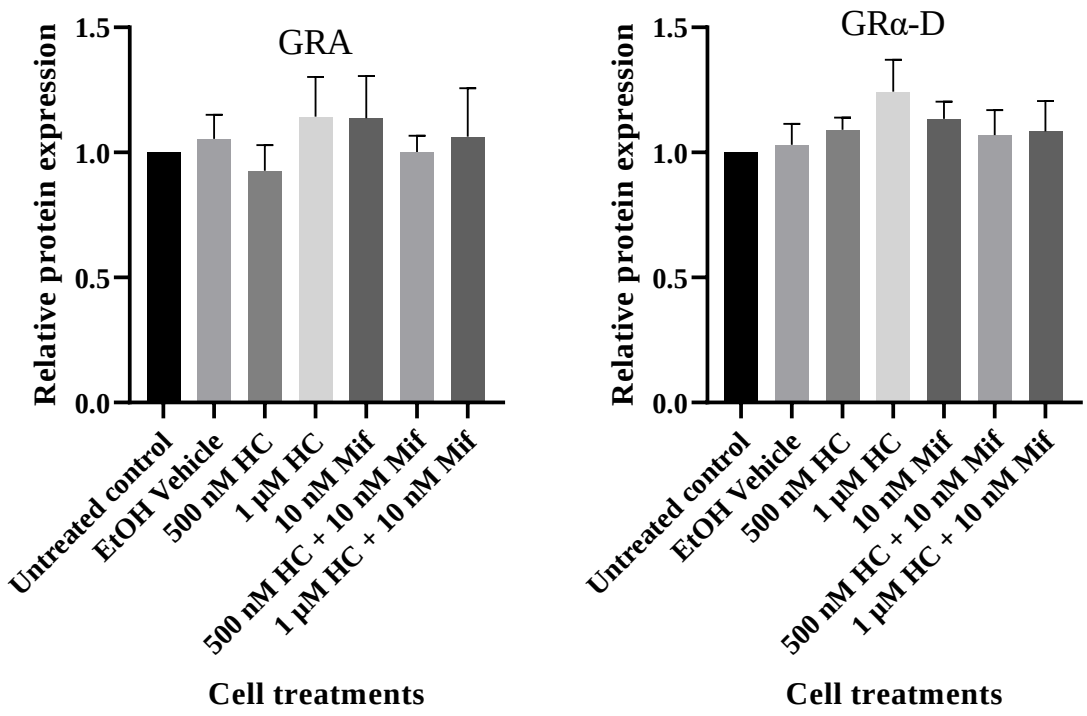


Figure 3.2. **Relative GRα-A, GRβ, GRγ, GRα-C, GRP, GRA, and GRα-D protein expression in SWAN-71 cells after 24-hour treatment with hydrocortisone and mifepristone as determined by Western blotting.** *Data presented as means ± SD, n = 3 for all treatment conditions carried out in technical duplicates. *** $p < 0.001$ compared to vehicle respective controls.*

Significant decreases in GRα-A protein expression was observed following treatment with TGF-β1 (5 ng/mL) (-30%, $p = 0.0071$) as well as TGF-β1 (5 ng/mL) in combination with hydrocortisone (1 μM) (-39%, $p = 0.0009$) compared to vehicle control (5×10^{-3} mM sodium citrate) (see Figures 3.3 and 3.4). In contrast, there were no significant differences in GR expression across all other isotypes following treatment for 24 hours. These results also demonstrate that TGF-β1 antagonist SB431542 reversed the TGF-β1-induced decreases in GRα-A protein expression.

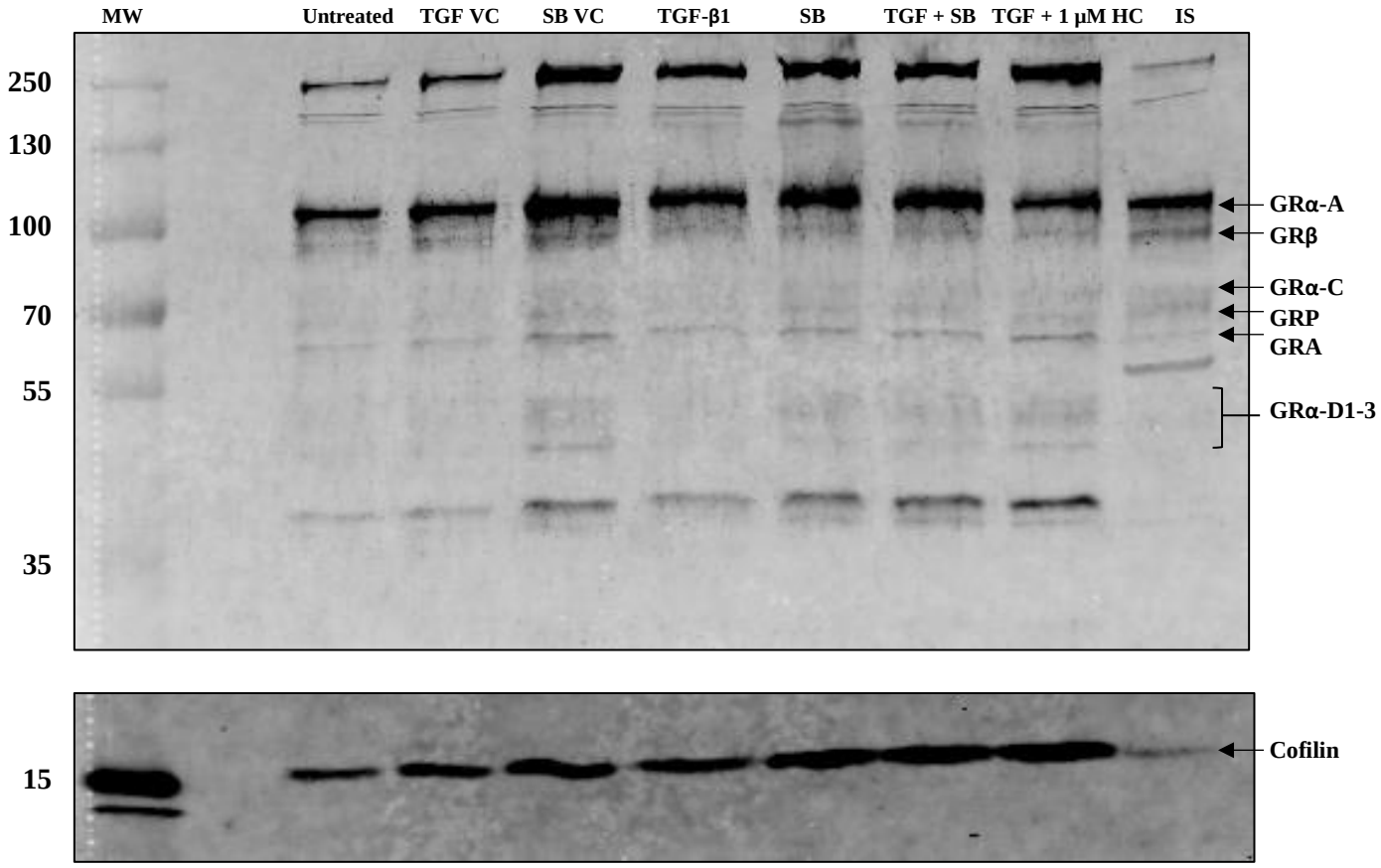
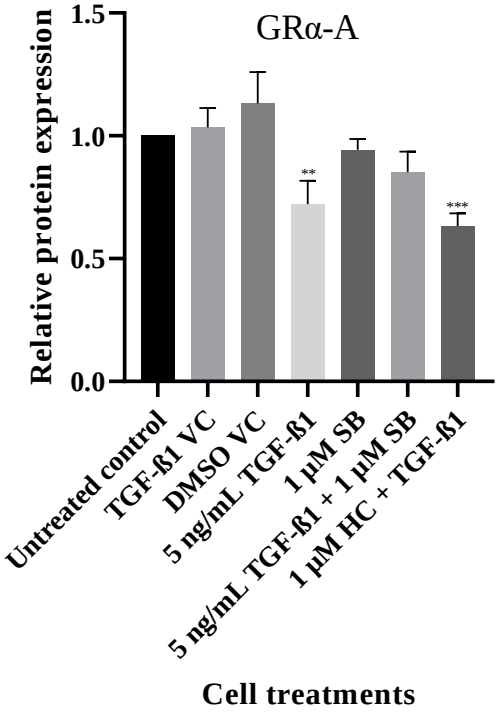
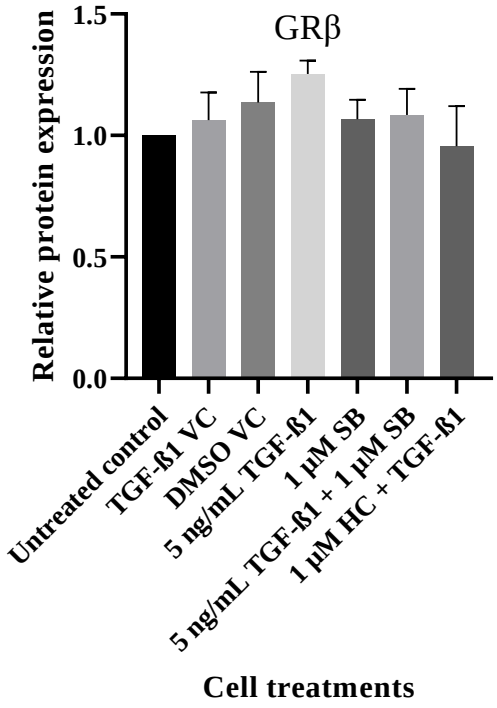
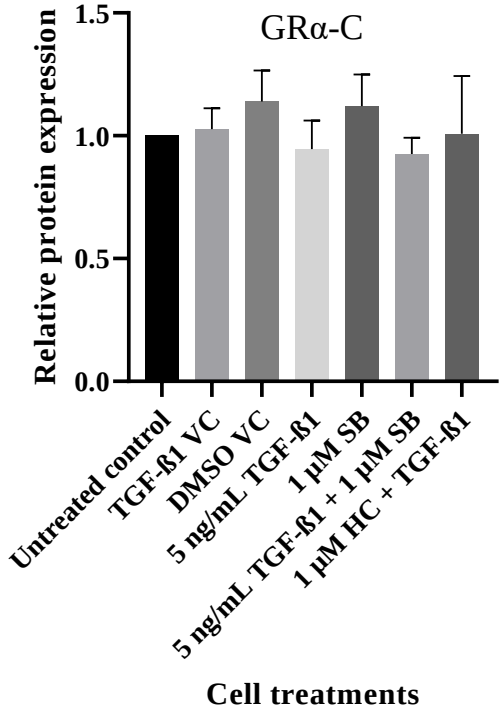


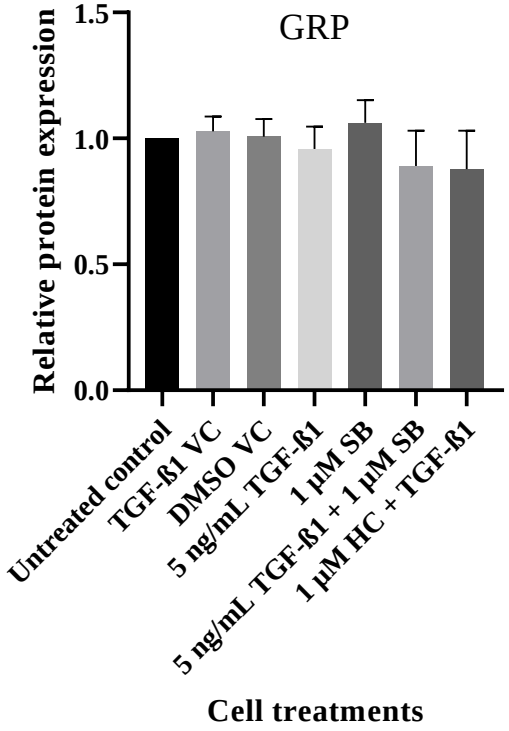
Figure 3.3. **Representative Western blot of GR protein expression in SWAN-71 cells after 24-hour treatment with TGF-β1 and SB431542.**

MW, molecular weight (kDa); VC, vehicle control; TGF, transforming growth factor-β1 (5 ng/mL); SB, SB431542; HC, hydrocortisone; IS, internal standard (A549 cell lysate).


GRα-A
Relative protein expression
1.5
1.0
0.5
0.0
**
***
Untreated control
TGF-ß1 VC
DMSO VC
5 ng/mL TGF-ß1
1 µM SB
5 ng/mL TGF-ß1 + 1 µM SB
1 µM HC + TGF-ß1
Cell treatments


GRβ
Relative protein expression
1.5
1.0
0.5
0.0
Untreated control
TGF-ß1 VC
DMSO VC
5 ng/mL TGF-ß1
1 µM SB
5 ng/mL TGF-ß1 + 1 µM SB
1 µM HC + TGF-ß1
Cell treatments


GRα-C
Relative protein expression
1.5
1.0
0.5
0.0
Untreated control
TGF-ß1 VC
DMSO VC
5 ng/mL TGF-ß1
1 µM SB
5 ng/mL TGF-ß1 + 1 µM SB
1 µM HC + TGF-ß1
Cell treatments


GRP
Relative protein expression
1.5
1.0
0.5
0.0
Untreated control
TGF-ß1 VC
DMSO VC
5 ng/mL TGF-ß1
1 µM SB
5 ng/mL TGF-ß1 + 1 µM SB
1 µM HC + TGF-ß1
Cell treatments

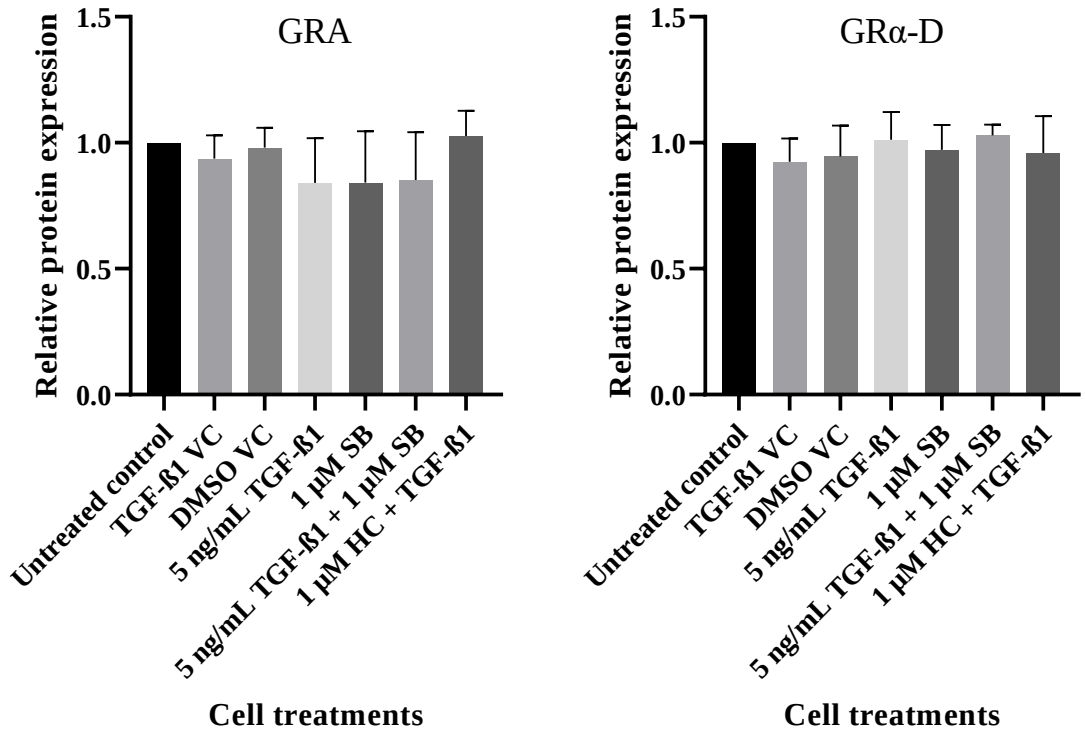


Figure 3.4. Relative GRα-A, GRβ, GRγ, GRα-C, GRP, GRA, and GRα-D protein expression in SWAN-71 cells after 24-hour treatment with TGF-β1 and SB431542 as determined by Western blotting. *Data presented as means ± SD, n = 3 for all treatment conditions carried out in technical duplicates. *** p < 0.001 and ** p < 0.01 compared to vehicle respective controls.*

3.4.2 Effect of 24-hour drug treatment on GR protein expression in JEG-3 cells using Western blot analysis

The total GR antibody (Bethyl Laboratories, Montgomery, TX, USA) detected approximately 13 specific bands in whole JEG-3 cell lysates (n = 3) after the cells were treated with hydrocortisone (500 nM or 1 µM), mifepristone (10 nM), TGF-β1 (5 ng/mL), or SB431542 (1 µM) for 24 hours. Among these bands, molecular weights of 95, 94, 91, 81, 74, 74, 65, and a cluster of blots between 50-55 kDa were observed that are consistent with known isoforms GRγ (95 kDa), GRα-A (94 kDa), GRβ (91 kDa), GRα-C (81 kDa), GRP (74 kDa), GRA (65 kDa), and GRα-D1-3 (50-55 kDa). Several unknown bands at molecular weights of 250, 130, 45, and 38 kDa were also observed.

A significant decrease in GRα-A protein expression was identified following 24-hour treatment with the highest concentration of hydrocortisone (1 µM) when compared to vehicle control (0.01% ethanol w/v) (-37%, p = 0.0011) (see Figures 3.5 and 3.6). No other significant differences were observed in other GR isotypes following hydrocortisone or mifepristone treatments. These results show that the GR receptor antagonist mifepristone (10 nM) reversed the effect of hydrocortisone (1 µM) following 24 hours incubation in JEG-3 cells.

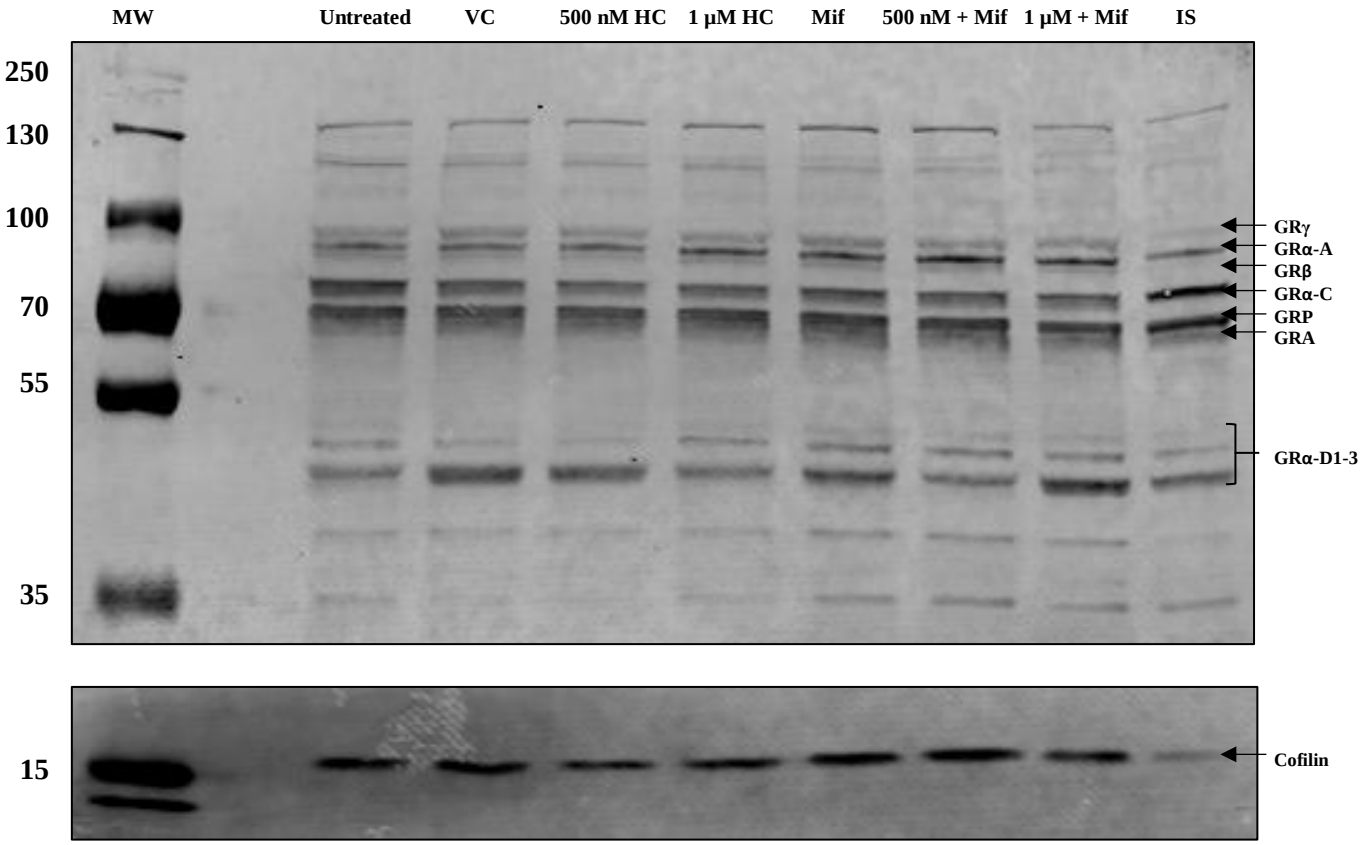
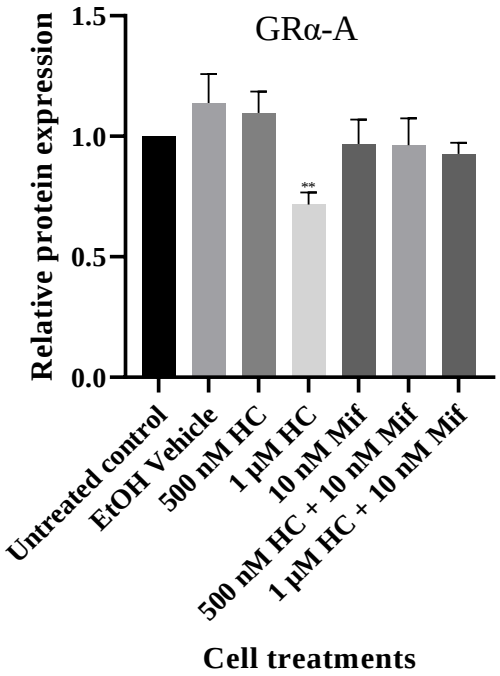
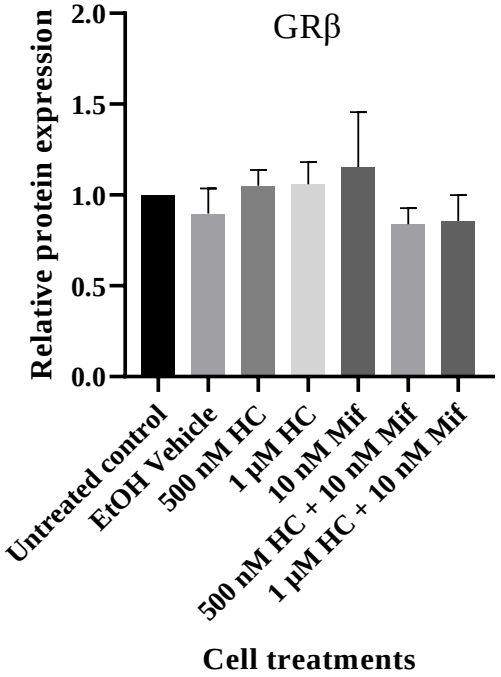
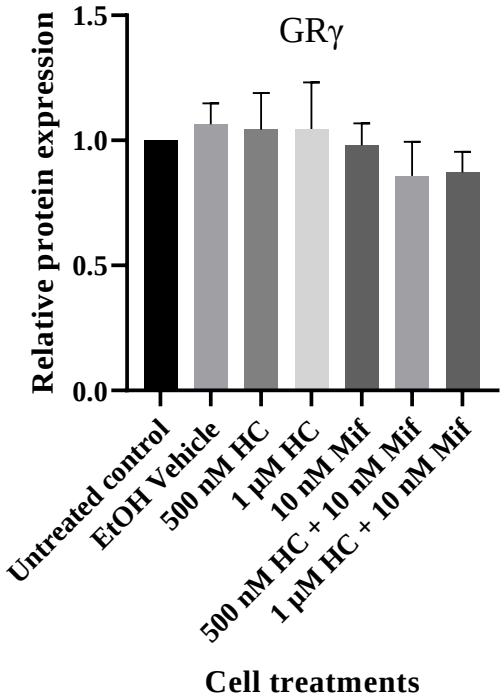


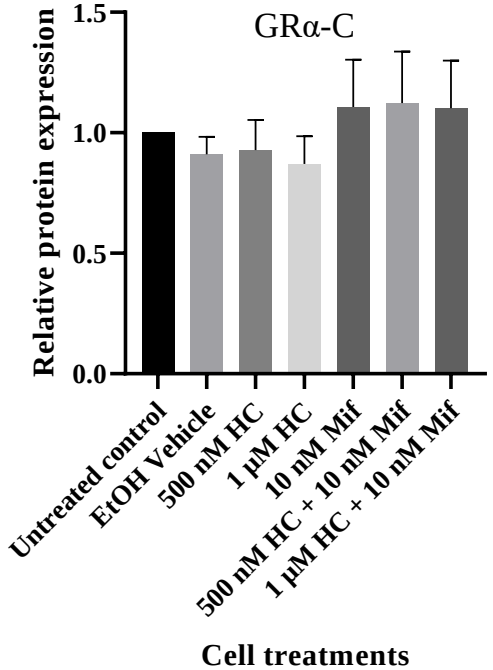
Figure 3.5. Representative Western blot of GR protein expression in JEG-3 cells after 24-hour treatment with hydrocortisone and mifepristone.

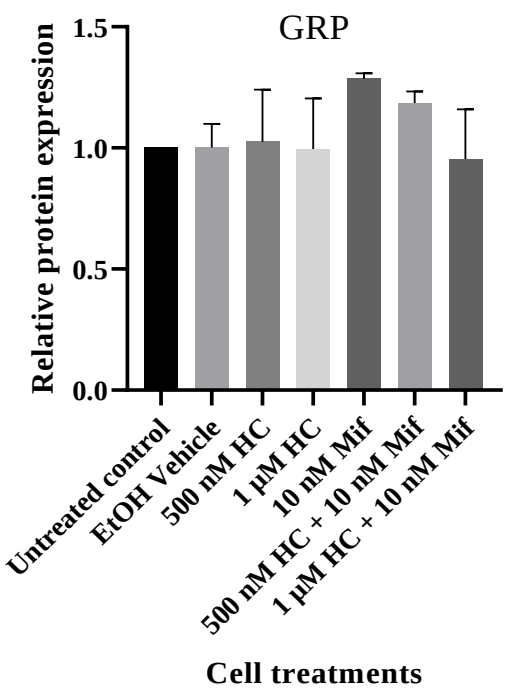
MW, molecular weight (kDa); VC, vehicle control; HC, hydrocortisone; Mif, mifepristone (10 nM); IS, internal standard (A549 cell lysate).

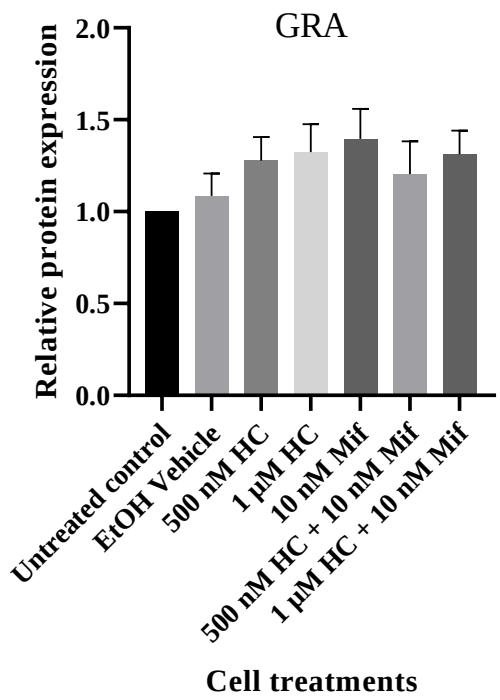

GRα-A
Relative protein expression
1.5
1.0
0.5
0.0
**
Untreated control
EtOH Vehicle
500 nM HC
1 µM HC
10 nM Mif
500 nM HC + 10 nM Mif
1 µM HC + 10 nM Mif
Cell treatments


GRβ
Relative protein expression
2.0
1.5
1.0
0.5
0.0
Untreated control
EtOH Vehicle
500 nM HC
1 µM HC
10 nM Mif
500 nM HC + 10 nM Mif
1 µM HC + 10 nM Mif
Cell treatments


GRγ
Relative protein expression
1.5
1.0
0.5
0.0
Untreated control
EtOH Vehicle
500 nM HC
1 µM HC
10 nM Mif
500 nM HC + 10 nM Mif
1 µM HC + 10 nM Mif
Cell treatments


GRα-C
Relative protein expression
1.5
1.0
0.5
0.0
Untreated control
EtOH Vehicle
500 nM HC
1 µM HC
10 nM Mif
500 nM HC + 10 nM Mif
1 µM HC + 10 nM Mif
Cell treatments


GRP
Relative protein expression
1.5
1.0
0.5
0.0
Untreated control
EtOH Vehicle
500 nM HC
1 µM HC
10 nM Mif
500 nM HC + 10 nM Mif
1 µM HC + 10 nM Mif
Cell treatments


GRA
Relative protein expression
2.0
1.5
1.0
0.5
0.0
Untreated control
EtOH Vehicle
500 nM HC
1 µM HC
10 nM Mif
500 nM HC + 10 nM Mif
1 µM HC + 10 nM Mif
Cell treatments

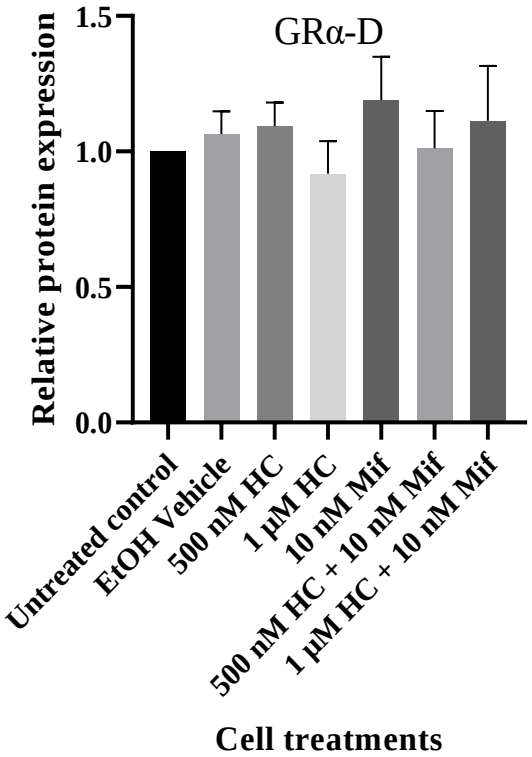

GRα-D
Relative protein expression
1.5
1.0
0.5
0.0
Untreated control
EtOH Vehicle
500 nM HC
1 µM HC
10 nM Mif
500 nM HC + 10 nM Mif
1 µM HC + 10 nM Mif
Cell treatments

Figure 3.6. Relative GRα-A, GRβ, GRγ, GRα-C, GRP, GRA, and GRα-D protein expression in JEG-3 cells after 24-hour treatment with hydrocortisone and mifepristone as determined by Western blotting. *Data presented as means ± SD, n = 3 for all treatment conditions carried out in technical duplicates. ** $p < 0.01$ compared to vehicle respective controls.*

Significant decreases were seen in GRα-A protein expression following TGF-β1 (5 ng/mL) and TGF-β1 (5 ng/mL) combined with hydrocortisone (1 µM) compared to vehicle control (5 x 10^{-3} mM sodium citrate) (-32%, p = 0.0138 and -32%, p = 0.0125, respectively. (see Figures 3.7 and 3.8). There was also a significant increase in GRβ protein expression after 24-hour treatment with TGF-β1 compared to vehicle control (1.69-fold, p = 0.0008) (see Figures 3.7 and 3.8). There were no other significant differences observed between GR isotype protein expression following pharmacological treatments. These results also demonstrate that the TGF-β1 antagonist SB431542 reversed the TGF-β1-induced changes in GRα-A and GRβ protein expression in JEG-3 cells.

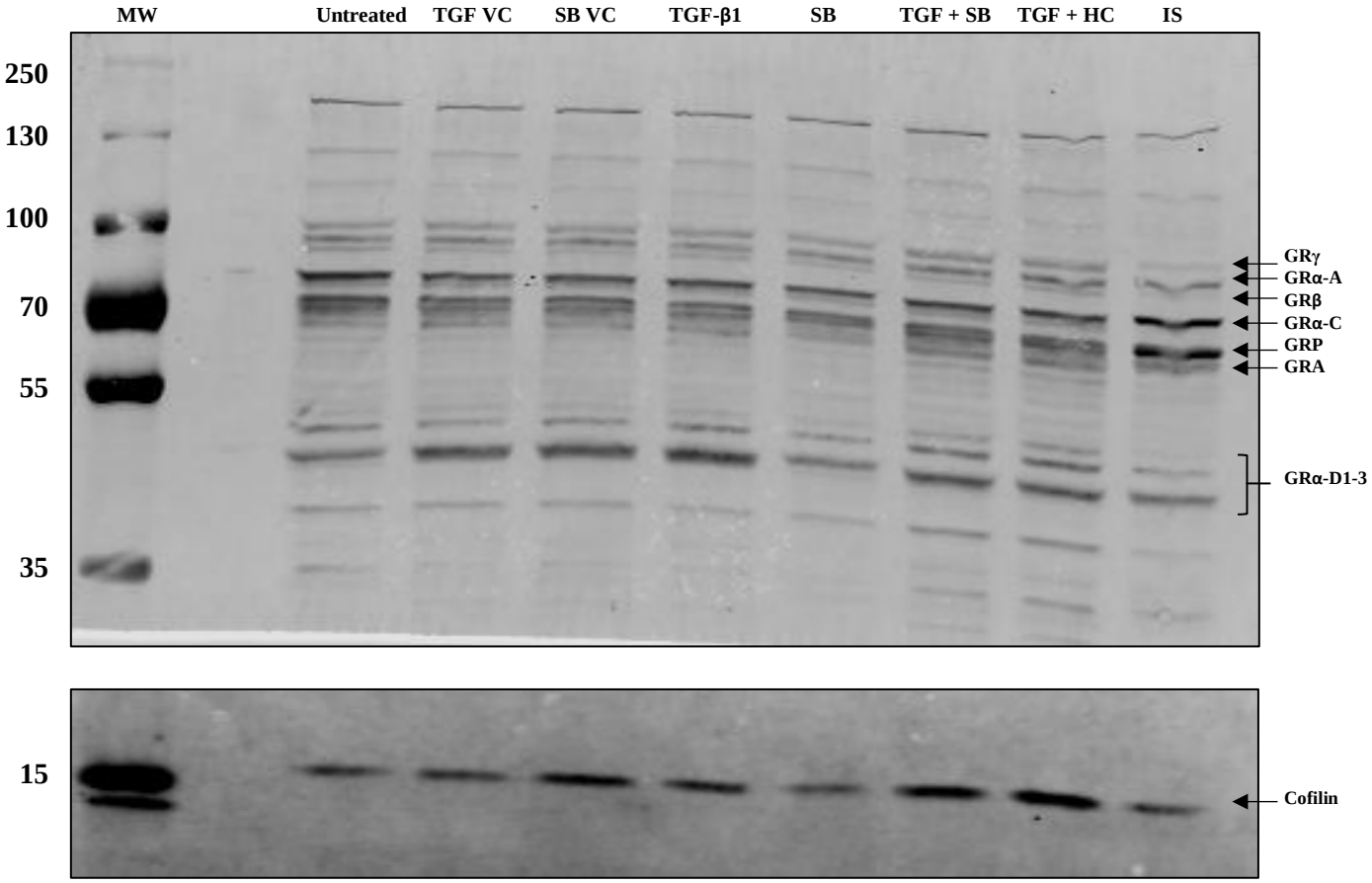
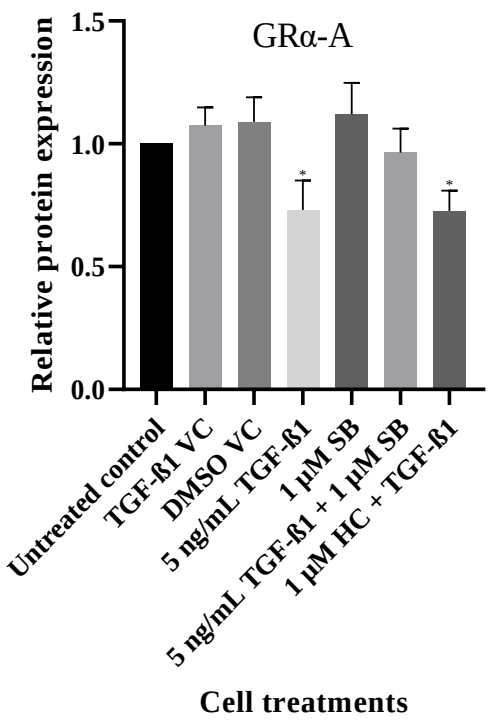
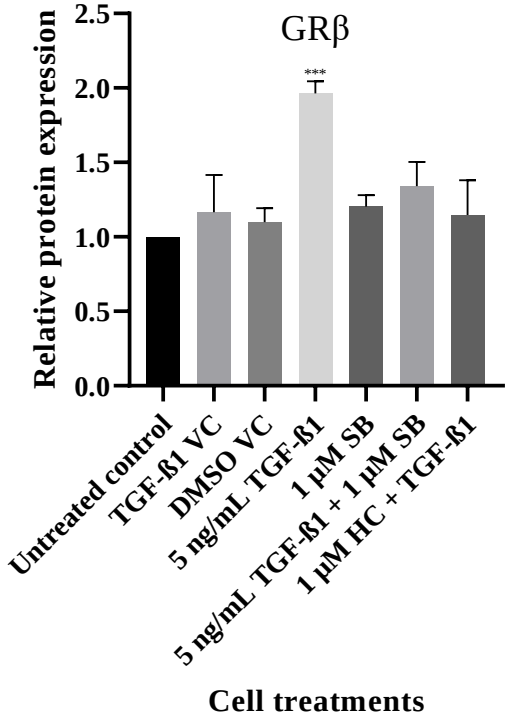
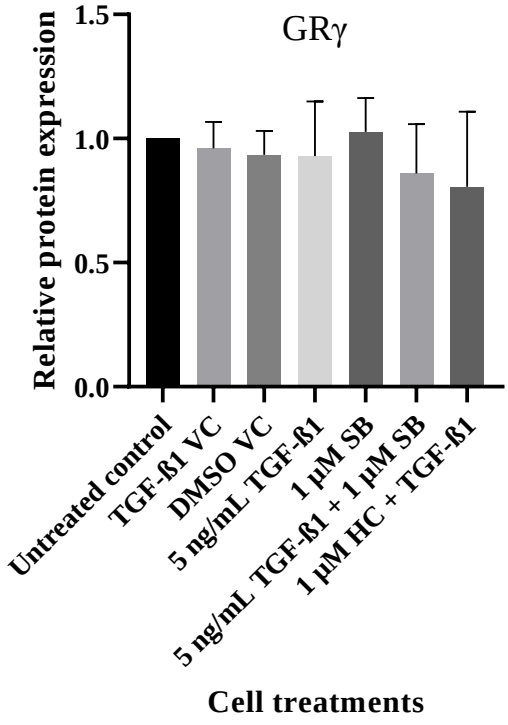


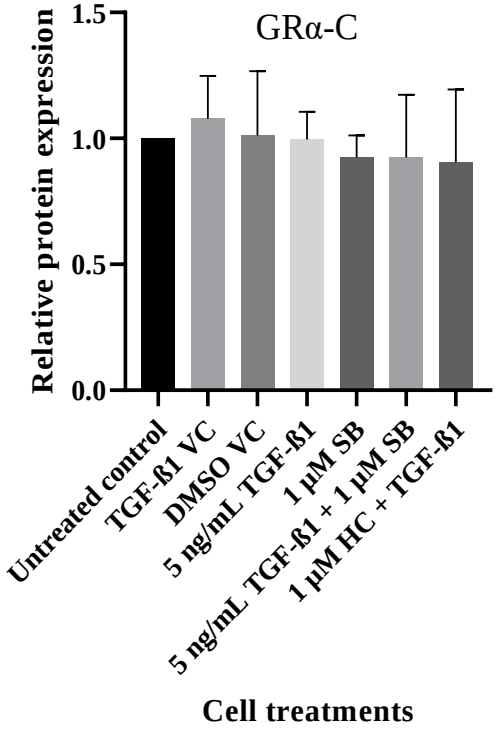
Figure 3.7. Representative Western blot of GR protein expression in JEG-3 cells after 24-hour treatment with TGF-β1 and SB431542.

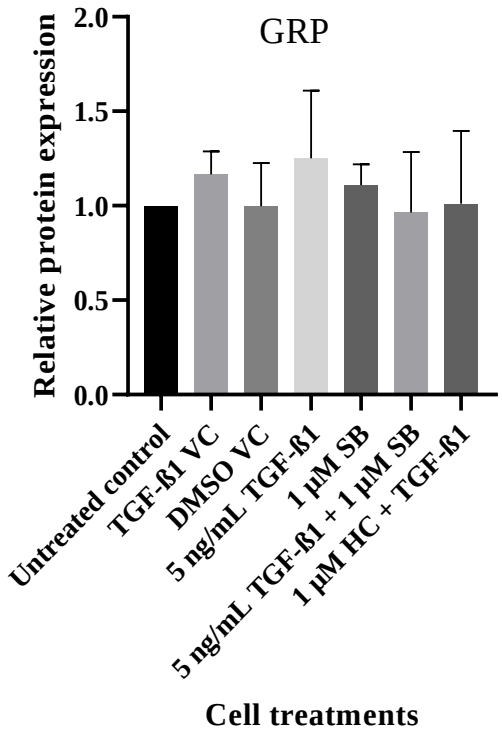
MW, molecular weight (kDa); VC, vehicle control; TGF, transforming growth factor-β1 (5 ng/mL); SB, SB431542; HC, hydrocortisone; IS, internal standard (A549 cell lysate).

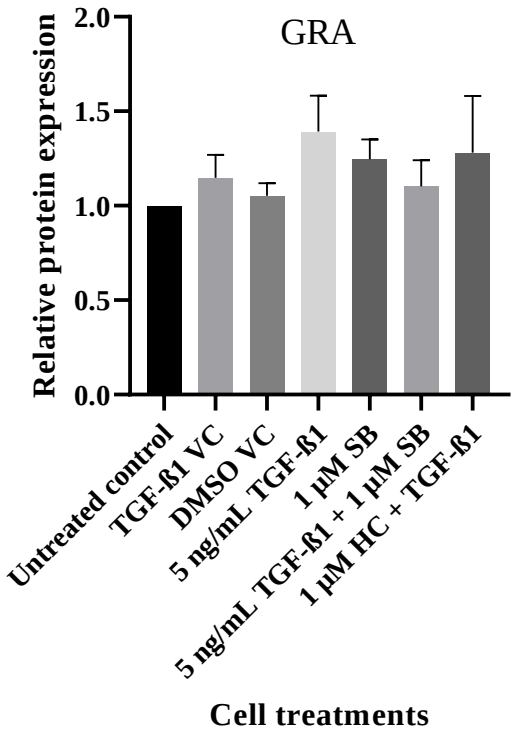

GRα-A
Relative protein expression
1.5
1.0
0.5
0.0
*
*
Untreated control
TGF-ß1 VC
DMSO VC
5 ng/mL TGF-ß1
1 µM SB
5 ng/mL TGF-ß1 + 1 µM SB
1 µM HC + TGF-ß1
Cell treatments


GRβ
Relative protein expression
2.5
2.0
1.5
1.0
0.5
0.0
***
Untreated control
TGF-ß1 VC
DMSO VC
5 ng/mL TGF-ß1
1 µM SB
5 ng/mL TGF-ß1 + 1 µM SB
1 µM HC + TGF-ß1
Cell treatments


GRγ
Relative protein expression
1.5
1.0
0.5
0.0
Untreated control
TGF-ß1 VC
DMSO VC
5 ng/mL TGF-ß1
1 µM SB
5 ng/mL TGF-ß1 + 1 µM SB
1 µM HC + TGF-ß1
Cell treatments


GRα-C
Relative protein expression
1.5
1.0
0.5
0.0
Untreated control
TGF-ß1 VC
DMSO VC
5 ng/mL TGF-ß1
1 µM SB
5 ng/mL TGF-ß1 + 1 µM SB
1 µM HC + TGF-ß1
Cell treatments


GRP
Relative protein expression
2.0
1.5
1.0
0.5
0.0
Untreated control
TGF-ß1 VC
DMSO VC
5 ng/mL TGF-ß1
1 µM SB
5 ng/mL TGF-ß1 + 1 µM SB
1 µM HC + TGF-ß1
Cell treatments


GRA
Relative protein expression
2.0
1.5
1.0
0.5
0.0
Untreated control
TGF-ß1 VC
DMSO VC
5 ng/mL TGF-ß1
1 µM SB
5 ng/mL TGF-ß1 + 1 µM SB
1 µM HC + TGF-ß1
Cell treatments

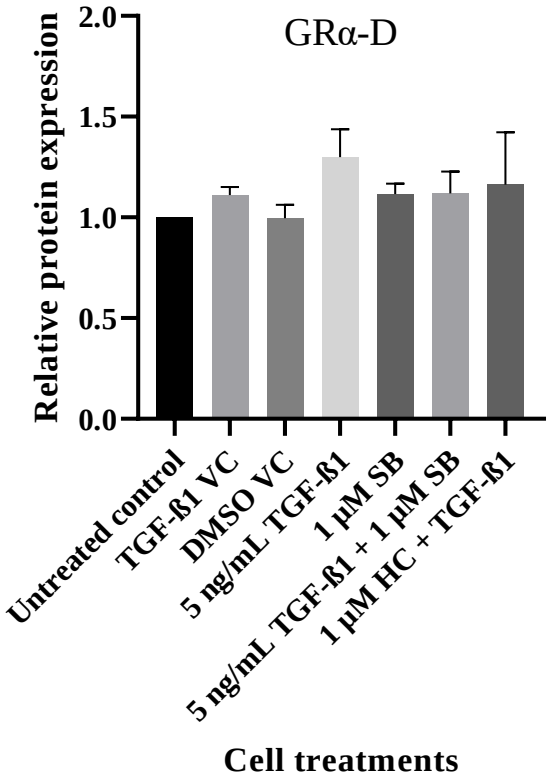

GRα-D
Relative protein expression
2.0
1.5
1.0
0.5
0.0
Untreated control
TGF-ß1 VC
DMSO VC
5 ng/mL TGF-ß1
1 µM SB
5 ng/mL TGF-ß1 + 1 µM SB
1 µM HC + TGF-ß1
Cell treatments

Figure 3.8. Relative GRα-A, GRβ, GRγ, GRα-C, GRP, GRA, and GRα-D protein expression in JEG-3 cells after 24-hour treatment with TGF-β1 and SB431542 as determined by Western blotting. *Data presented as means ± SD, n = 3 for all treatment conditions carried out in technical duplicates. *** $p < 0.001$, * $p < 0.05$ compared to vehicle respective controls.*

3.4.3 Effect of 24-hour drug treatment on SERT protein expression in SWAN-71 cells using Western blot analysis

Use of the SERT (SLC6A4) primary antibody (Thermo Fisher Scientific, Waltham, MA, USA) identified 5 specific bands in whole SWAN-71 cell lysates (n = 3) after cells were treated with hydrocortisone (500 nM or 1 µM), mifepristone (10 nM), TGF-β1 (5 ng/mL), or SB431542 (1 µM) for 24 hours. Among these bands, molecular weights of 150, 90, 80, 68, and 50 kDa. Native SERT has been shown to be ~68 kDa and this was the band used for subsequent analysis (330, 331). A significant increase in SERT protein expression was observed following 24-hour treatment with hydrocortisone (1 µM) compared to vehicle control (0.01% ethanol w/v) (1.48-fold, p = 0.0204) (see Figures 3.9 and 3.10). No statistically significant differences were identified for mifepristone (10 nM) nor hydrocortisone (500 nM or 1 µM) and mifepristone (10 nM) combined treatment groups. With respect to TGF-β1 (5 ng/mL) treatment groups, significant increases in SERT protein expression were similarly observed in both TGF-β1 and TGF-β1 combined with hydrocortisone (1 µM) when compared to vehicle control (0.05 mM sodium citrate) (1.39-fold, p = 0.0387 and 1.38-fold, p = 0.0479, respectively) (see Figures 3.11 and 3.12). There were no significant alterations in SERT protein expression for the SB431542 (1 µM) treatment groups. These results show that hydrocortisone- or TGF-β1-induced decreases in SERT protein expression were reversed by selective inhibitors for these compounds in SWAN-71 cells. No additive effects were observed with a combination of hydrocortisone (1 µM) and TGF-β1 (5 ng/mL) following 24 hours incubation in SWAN-71 cells.

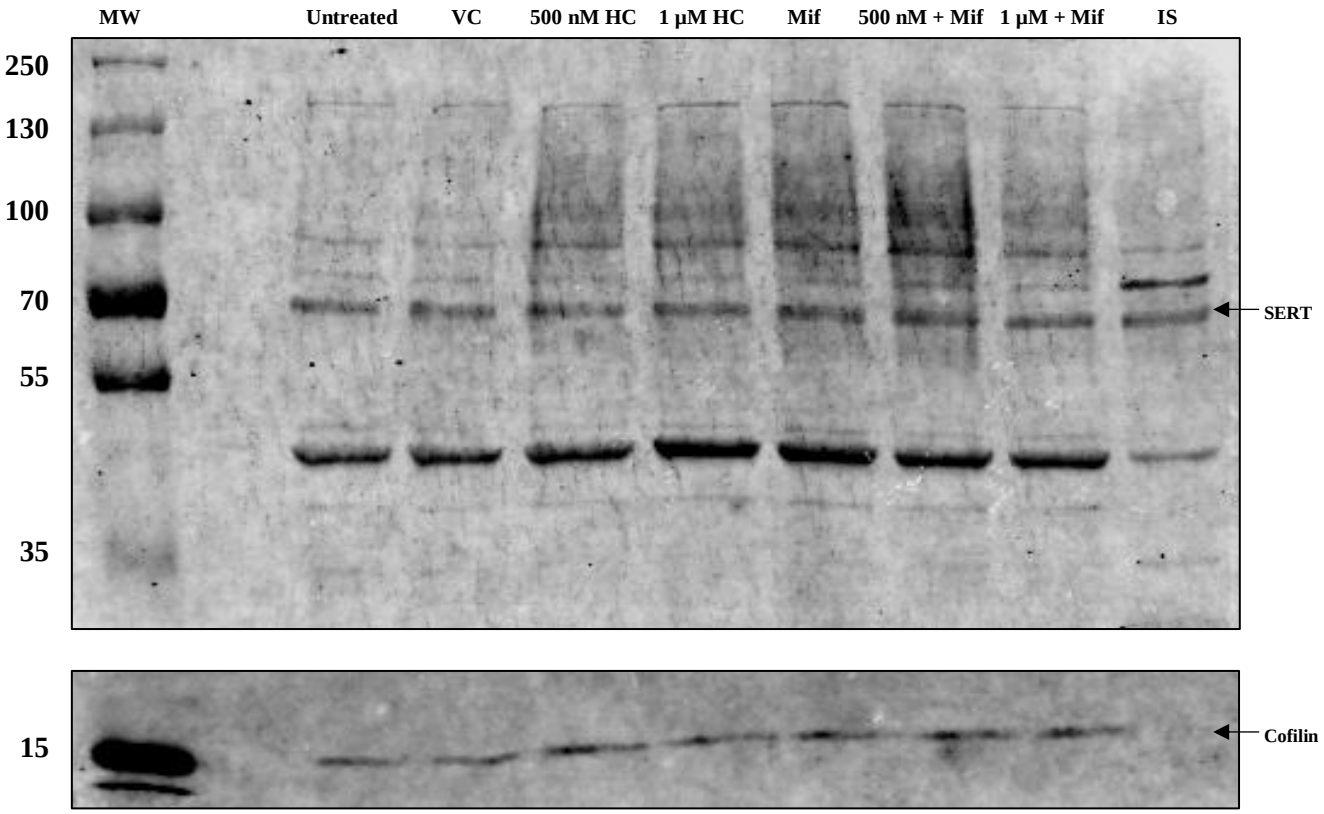


Figure 3.9. Representative Western blot of SERT protein expression in SWAN-71 cells after 24-hour treatment with hydrocortisone and mifepristone.

MW, molecular weight (kDa); VC, vehicle control; HC, hydrocortisone; Mif, mifepristone (10 nM); IS, internal standard (A549 cell lysate).

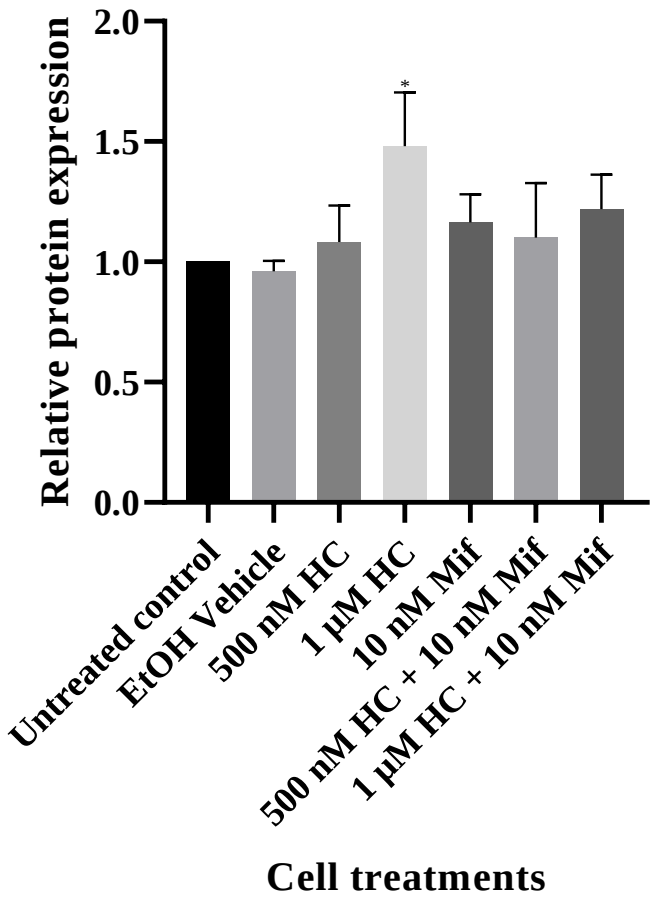


Figure 3.10. Relative SERT protein expression in SWAN-71 cells after 24-hour treatment with hydrocortisone and mifepristone as determined by Western blotting. *Data presented as means ± SD, n = 3 for all treatment conditions carried out in technical duplicates. * p < 0.05 compared to vehicle control.*

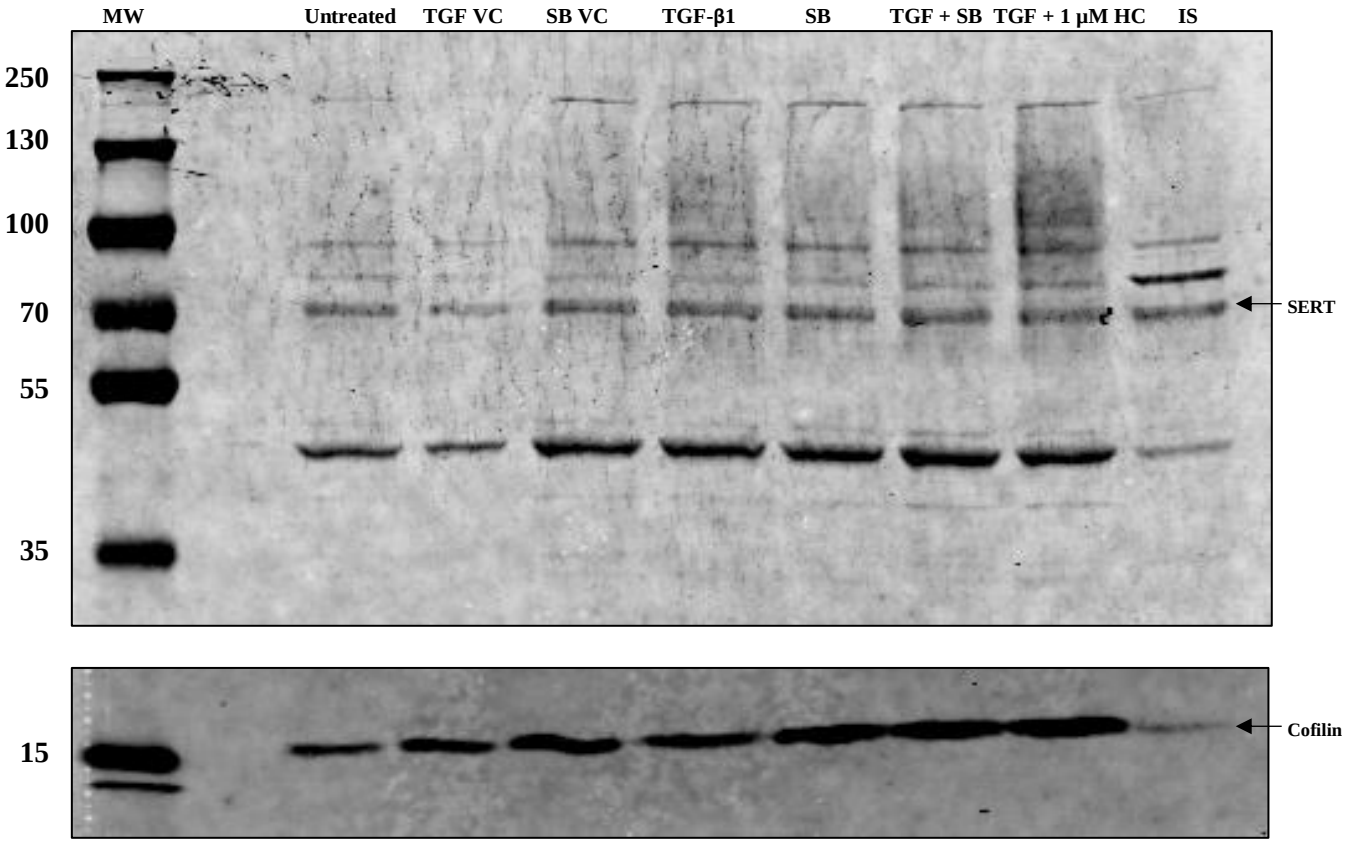


Figure 3.11. Representative Western blot of SERT protein expression in SWAN-71 cells after 24-hour treatment with TGF-β1 and SB431542.

MW, molecular weight (kDa); VC, vehicle control; TGF, transforming growth factor-β1 (5 ng/mL); SB, SB431542; HC, hydrocortisone; IS, internal standard (A549 cell lysate).

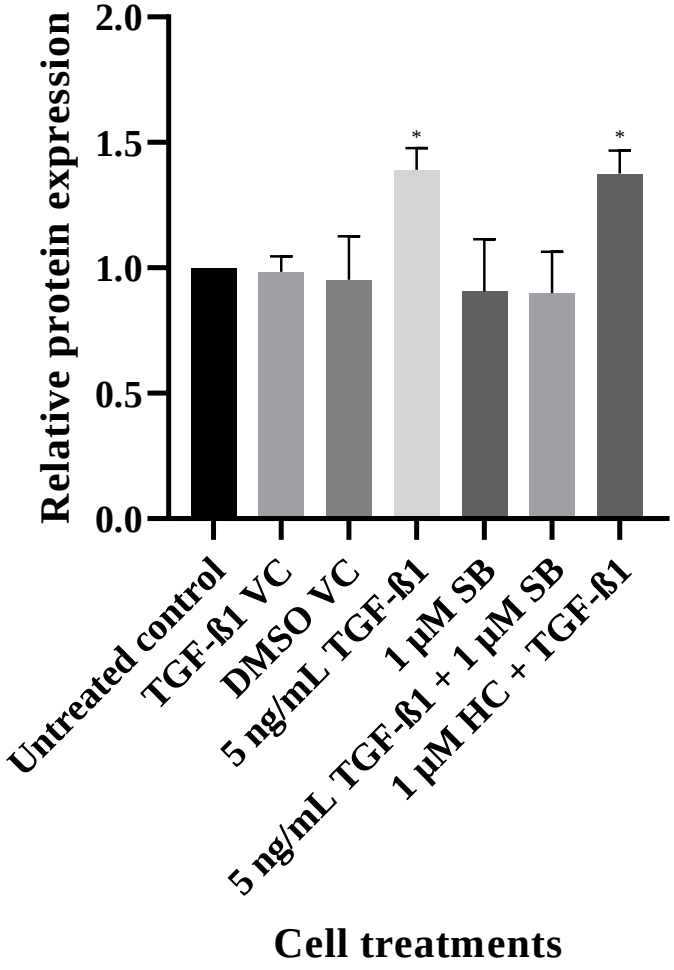


Figure 3.12. Relative SERT protein expression in SWAN-71 cells after 24-hour treatment with TGF-β1 and SB431542 as determined by Western blotting.

*Data presented as means ± SD, n = 3 for all treatment conditions carried out in technical duplicates. * p < 0.05 compared to respective vehicle controls.*

3.4.4 Effect of 24-hour drug treatment on SERT protein expression in JEG-3 cells using Western blot analysis

Use of the SERT primary antibody (Thermo Fisher Scientific, Waltham, MA, USA) identified 6 specific bands in whole JEG-3 cell lysates (n = 3) after treatment with hydrocortisone (500 nM or 1 µM), mifepristone (10 nM), TGF-β1 (5 ng/mL), or SB431542 (1 µM) for 24 hours. Among these bands, molecular weights of 150, 90, 80, 68, and 50-52 kDa. A significant increase in SERT protein expression was observed following hydrocortisone (1 µM) treatment compared to vehicle control (0.01% ethanol w/v) (1.43-fold, $p < 0.0001$) (see Figures 3.13 and 3.14). Similarly, significant increases in SERT protein expression were observed after TGF-β1 and TGF-β1 (5 ng/mL) combined with hydrocortisone (1 µM) compared to vehicle control (5 x 10^{-3} mM sodium citrate) (1.80-fold, $p < 0.0001$ and 1.84-fold, $p < 0.0001$, respectively) (see Figures 2.23 and 2.24). Further, a significant increase in SERT protein expression was also observed in TGF-β1 (5 ng/mL) combined with SB431542 (1 µM) compared to both TGF-β1 vehicle control and DMSO (0.5 mM, 0.01% w/v) (1.35-fold, $p = 0.0287$ and 1.34-fold, $p = 0.0355$, respectively) (see Figures 3.15 and 3.16). No statistically significant differences in SERT protein expression were observed in the hydrocortisone (500 nM), SB431542 (1 µM), nor the other combined treatment groups ($p > 0.05$). These results show that hydrocortisone- or TGF-β1-induced decreases in SERT expression were reversed by selective inhibitors for these compounds in JEG-3 cells. No additive effects were observed with a combination of hydrocortisone (1 µM) and TGF-β1 (5 ng/mL) following 24 hours incubation in JEG-3 cells.

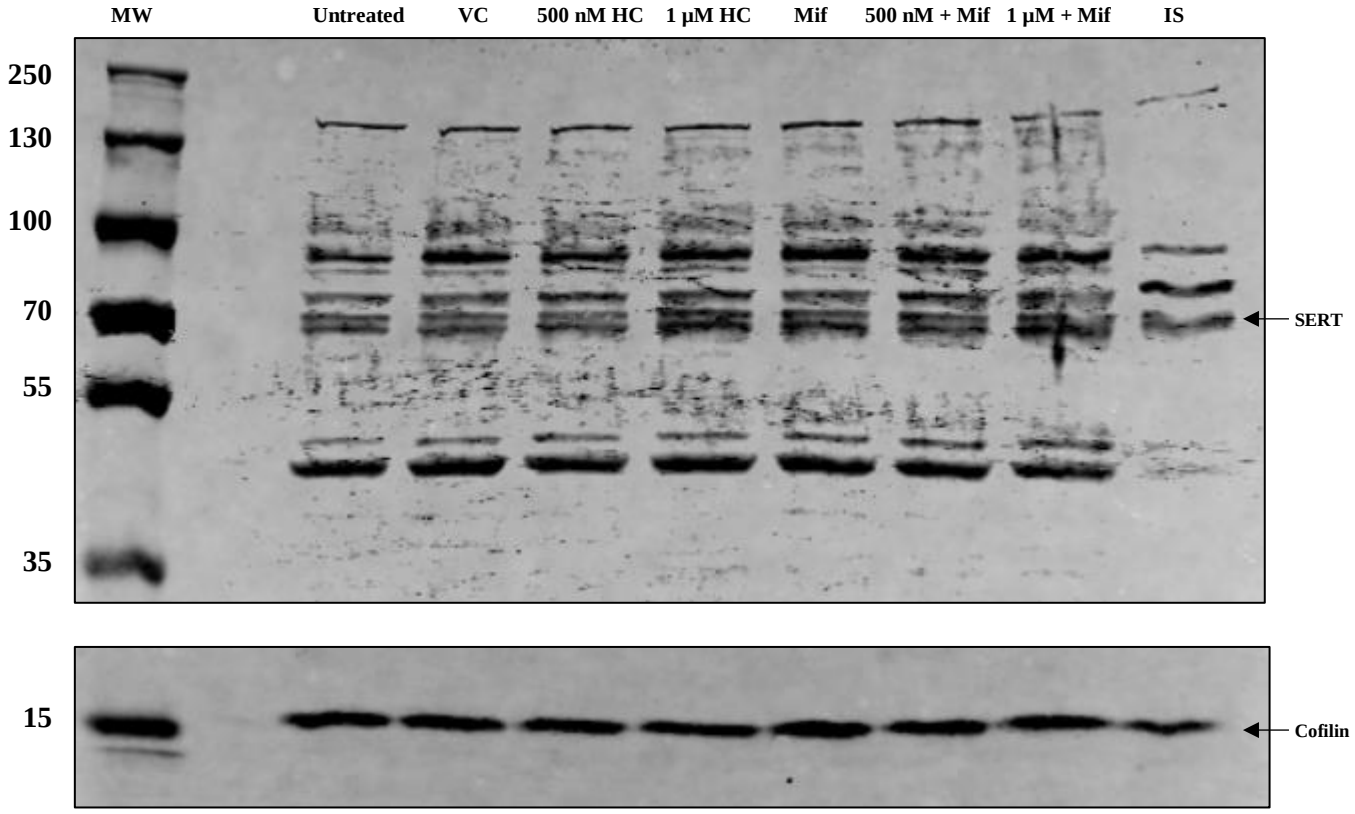


Figure 3.13. Representative Western blot of SERT protein expression in JEG-3 cells after 24-hour treatment with hydrocortisone and mifepristone.

MW, molecular weight (kDa); VC, vehicle control; HC, hydrocortisone; Mif, mifepristone (10 nM); IS, internal standard (A549 cell lysate).

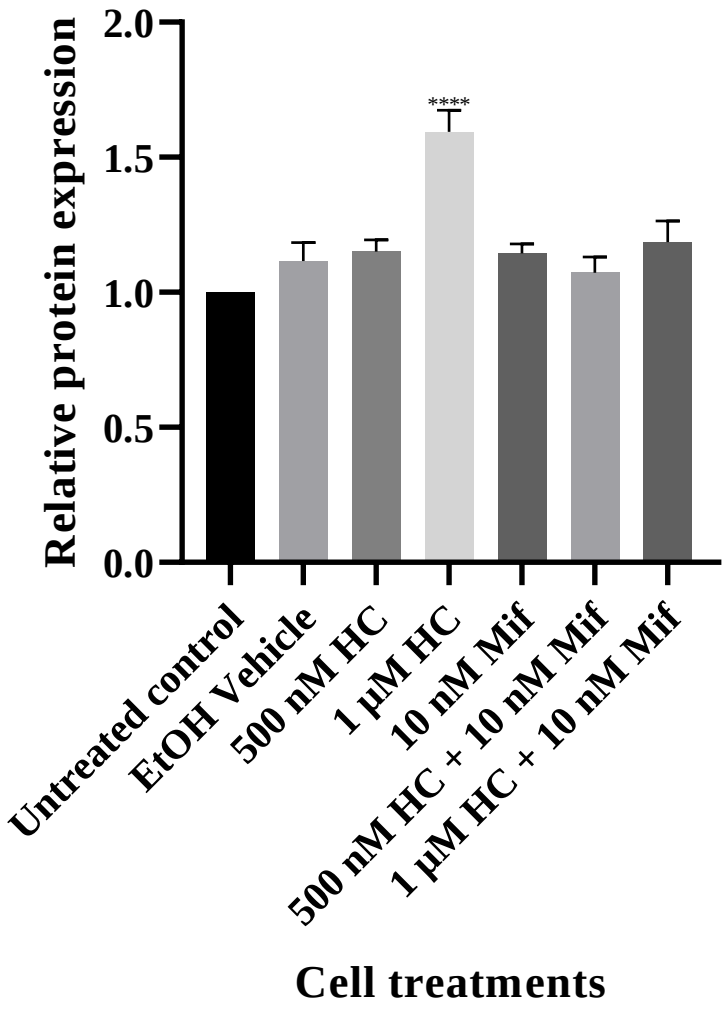


Figure 3.14. Relative SERT protein expression in JEG-3 cells after 24-hour treatment with hydrocortisone and mifepristone as determined by Western blotting. *Data presented as means ± SD, n = 3 for all treatment conditions carried out in technical duplicates. **** $p < 0.0001$ compared to vehicle control.*

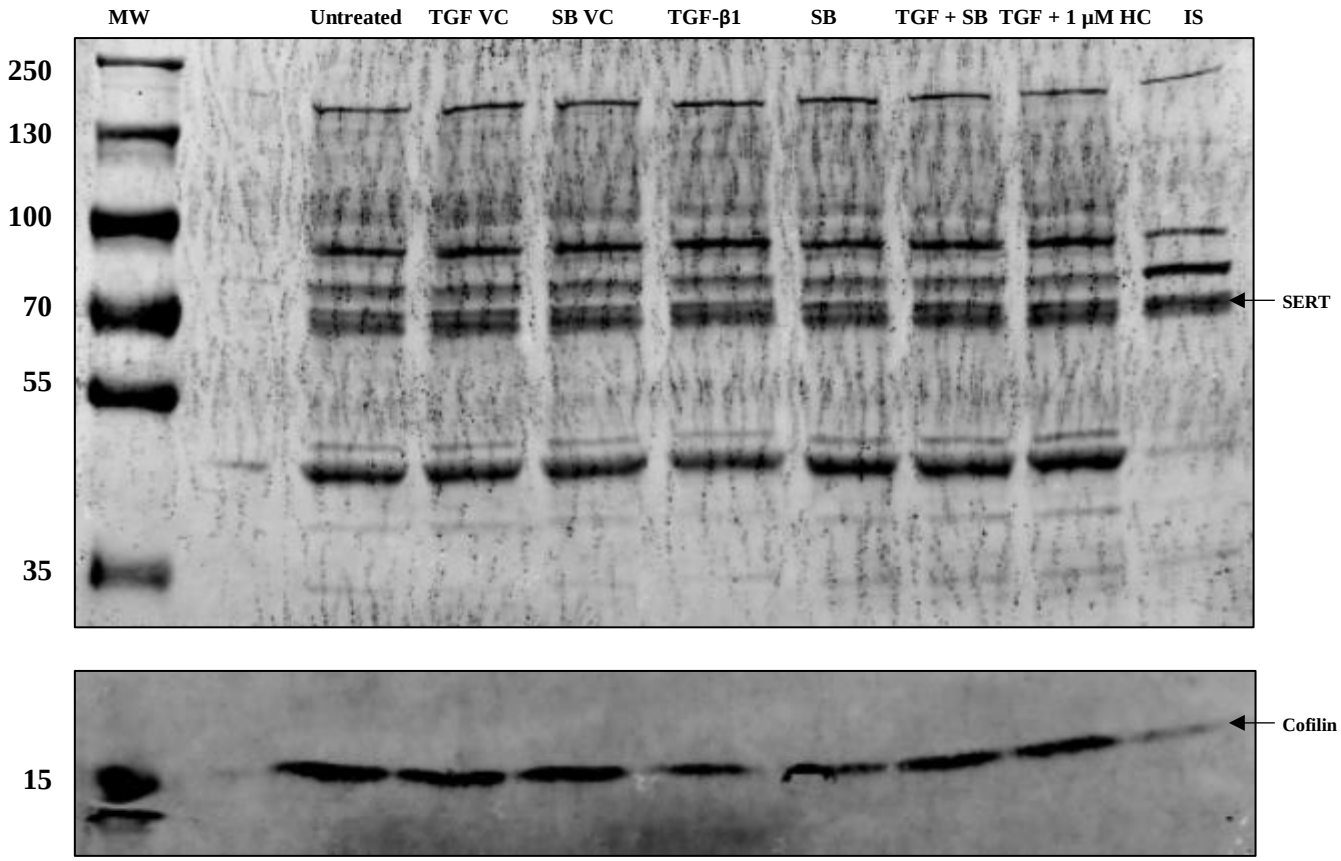


Figure 3.15. Representative Western blot of SERT protein expression in JEG-3 cells after 24-hour treatment with TGF-β1 and SB431542.

MW, molecular weight (kDa); VC, vehicle control; TGF, transforming growth factor-β1 (5 ng/mL); SB, SB431542; HC, hydrocortisone; IS, internal standard (A549 cell lysate).

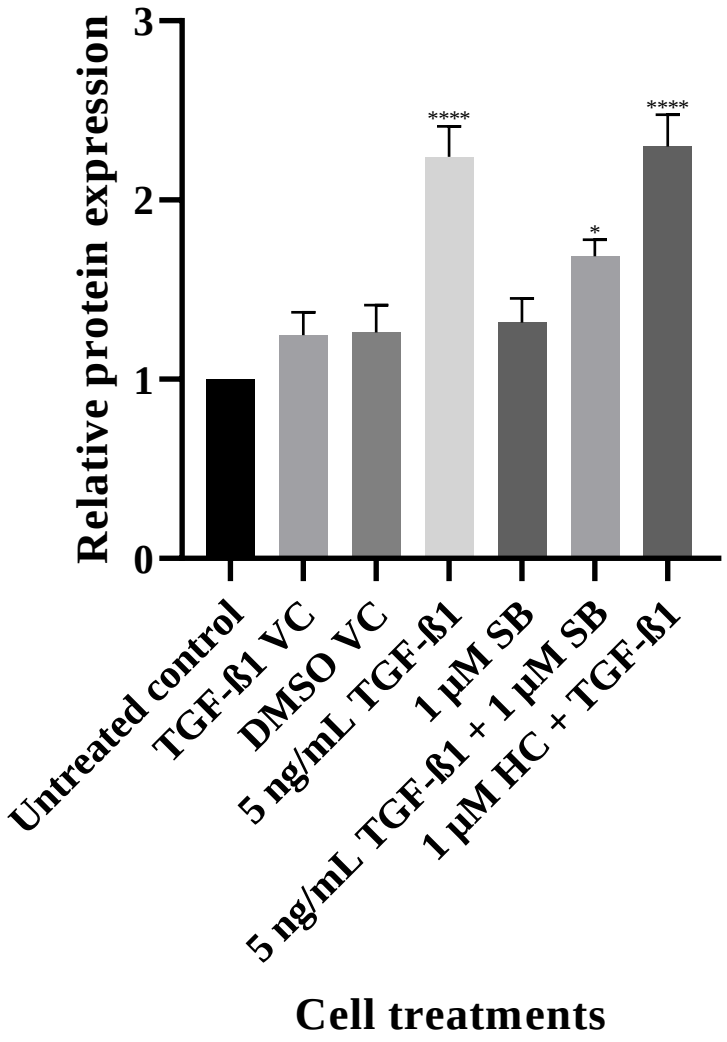


Figure 3.16. Relative SERT protein expression in JEG-3 cells after 24-hour treatment with TGF-β1 and SB431542 as determined by Western blotting. *Data presented as means ± SD, n = 3 for all treatment conditions carried out in technical duplicates. **** $p < 0.0001$, * $p < 0.05$ compared to respective vehicle controls.*

3.4.5 Effect of 24-hour drug treatment on GR mRNA expression in SWAN-71 cells using NanoString nCounter analysis

To quantitate gene expression of the GR, the present study used custom NanoString nCounter probes that target transcripts on all GR isotypes (NanoString Technologies, Seattle, WA). Using this highly sensitive tool, total GR mRNA expression in whole SWAN-71 cell lysates (n = 3) after treatment with hydrocortisone (1 μM), mifepristone, TGF-β1 (5 ng/mL), or SB431542 (1 μM) for 24 hours. There were no statistically significant differences between the vehicle control and the untreated control groups ($p > 0.05$) following 24 hours incubation, indicating that the vehicle controls (ethanol, DMSO, and sodium citrate) had no effect on total GR mRNA expression (see Figure 3.17). There were also no statistically significant alterations in GR mRNA expression following pharmacological treatment for 24 hours ($p > 0.05$). These results show that neither hydrocortisone (1 μM), TGF-β1 (5 ng/mL), nor SB431542 (1 μM) had any effect on GR mRNA expression following 24 hours incubation in SWAN-71 cells.

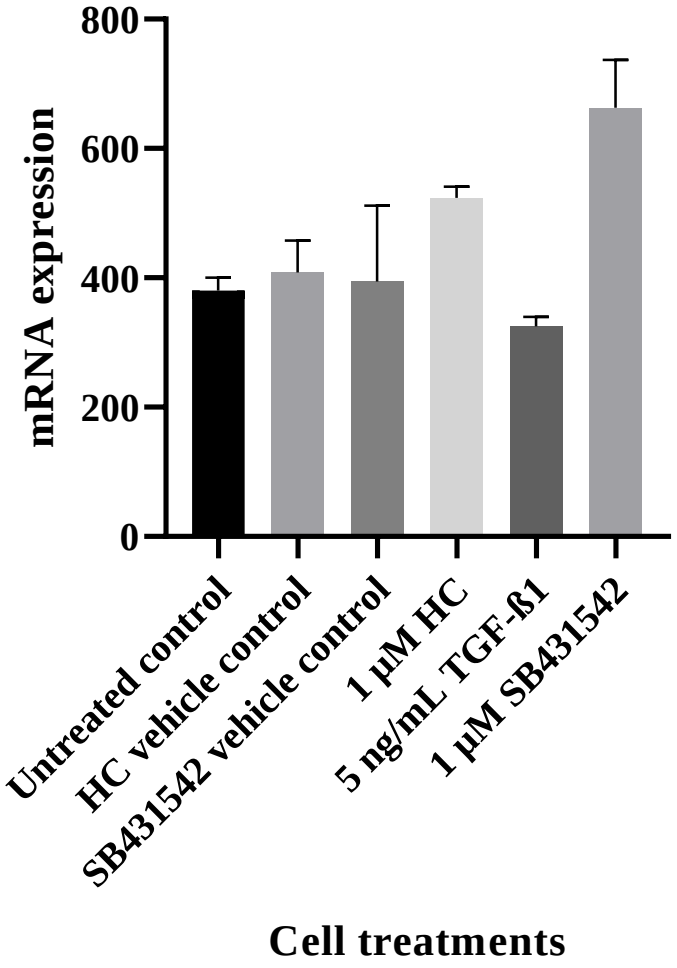


Figure 3.17. GR mRNA expression in SWAN-71 cells after 24-hour treatment with hydrocortisone, TGF-β1, and SB431542 as determined by NanoString nCounter.

Data presented as means ± SD, n = 3 for all treatment conditions.

3.5 Discussion

Although pregnancy-related pathologies such as PE, IUGR, and PTB continue to burden all human societies, their underlying aetiologies remain elusive. While perturbations in GC, TGF-β1, and serotonin signalling mechanisms are frequently associated with gestational abnormalities, their relationships have seldom been investigated in the placenta. The aim of the present study was to elucidate the effects of supraphysiological concentrations of cortisol and TGF-β1 on SWAN-71 and JEG-3 placental cell model viability through the utilisation of MTT and LDH assays and to measure changes in GR isotype and SERT protein expression as determined by Western blotting.

3.5.1 Protein expression GR isotypes using Western blot analysis

The present study investigated GR isotypes protein expression in placental cell lines, SWAN-71 and JEG-3, after treatment with hydrocortisone, mifepristone, TGF-β1, or SB431542 for 24 hours through Western blot analysis. Several distinct bands with weights of 95, 94, 91, 81, 74, 65, 50-55, 48, and 38 kDa were identified in the GR Western blot analysis in the present study. According to the literature, the GR antibody (Bethyl Laboratories, Montgomery, TX, USA) has been previously characterised in peer-reviewed journals and shown to bind to a number of GR receptor variant isoforms in placental tissue, including GRγ (95 kDa), GRα-A (94 kDa), GRβ (91 kDa), GRα-C (81 kDa), GR-P (74 kDa), GR-A (65 kDa), and GRα-D1-3 (50-55 kDa), as well as unknown bands at 48 and 38 kDa (122, 124). These bands were quantitated in both SWAN-71 and JEG-3 cell lines for subsequent analysis. The A549 cell samples were used as internal standards for comparisons between gels and positive controls as they have been previously shown to express GR (304, 332, 333). Data were presented as protein expression relative to untreated controls groups.

Excess hydrocortisone, represented by the 1 μM treatment group, was shown to significantly decrease GRα-A protein expression in both SWAN-71 and JEG-3 cell lines. These alterations in GRα-A protein expression were reversed by the GR antagonist mifepristone (10 nM). Although the present study analysed whole-cell lysates, this appears to counter previous research with glucocorticoid-treated murine placentae noting significant GRα-A increases in cytoplasmic and nuclear fractions in both sexes (334, 335). In contrast, there were no other significant alterations in GR isotype protein expression after combined treatment with hydrocortisone and mifepristone. This decrease in GRα-A is significant as most gene expression occurs through the GRα-A isoform and is responsible for the majority of GR agonistic activity (83, 336). It is worth noting that although GRβ protein expression was observed to increase after 1 μM hydrocortisone treatment in both cell lines, this increase was not statistically significant. The human GRβ isoform does not bind glucocorticoid ligands nor induce transcription via GREs (88, 89, 337). Predominantly residing in the nucleus, GRβ acts as a dominant negative inhibitor of GRα-A and has been shown to antagonise glucocorticoid-mediated changes in various physiological properties across many cell types (338). It is evident in the present study that the observed increases, although insignificant, could be explained by the inhibition of the action of GRα-A by GRβ. Indeed, nuclear GRβ has been shown to increase in pregnant guinea pigs treated with betamethasone, irrespective of fetal sex or gestational age (335). Moreover, GRγ was not identified in the SWAN-71 cell line and protein expression did not change after the 24-hour pharmacological treatments in JEG-3. Similarly, GRP, GRA, GRα-C, and GRα-D1-3 isotype protein expression demonstrated no alterations following treatments in both cell lines. These results neither support nor counter previous findings of GRA and GRP increases in murine placentae post-dexamethasone treatment (334). According

to the literature, GRγ, GRA, and GRP may still have a role in mediating GR-induced transactivation of target genes despite exhibiting relatively low transactivity themselves (123, 339, 340). A reduction in GRP mRNA has also been hypothesised to decrease the responsiveness of trophoblasts to GCs due to its role in regulating GRα-mediated transcriptional activity and warrants further investigation (85, 341).

Perturbations in GR transactivity have been associated with placental pathology (122, 123). In the literature, excess cortisol has been demonstrated to decrease GR mRNA and protein expression (92, 93) and these findings are supported by the present study with particular respect to GRα-A. The underlying mechanisms remain equivocal, however. It has been proposed that a reduction in GRα-A might be due to ligand-induced downregulation at the level of GR protein or GR mRNA including repression of GR promoter activity, decreased stability of GR mRNA, and/or increased proteasomal degradation of the GR (85, 342-347). Previous studies have also indicated that supraphysiological GCs may affect trophoblast function by inhibiting the expression of important invasion-related genes, MMP-2 and MMP-9 (149), or inhibit proliferation, migration, and invasion through the regulation of the *SERPINE1* gene (151). These theories warrant further investigation. Ultimately, the significant decreases in GRα-A expression, as a consequence of exposure to supraphysiological levels of cortisol as observed in the present study, may indicate GC resistance and this was consistent with previous research using other cell types (130, 332). It is important to note that findings of the present study bear little consequence to advancing our understanding of specific GR isotype transactivity as whole-cell lysates were analysed. In contrast to previous research, whole-cell GR isotype protein expression lacks significant explanatory power with respect to GR physiological processes compared to fractionated lysate studies (334, 335).

Significant decreases in GRα-A were similarly observed in both SWAN-71 and JEG-3 cell lines after treatment with TGF-β1 (5 ng/mL) and TGF-β1 (5 ng/mL) combined with hydrocortisone (1 μM) which were reversed by SMAD2/3 antagonist SB431542. These results support previous findings pertaining to impaired GC activity in A549 cells with decreased cellular levels of GRα-A (304). Moreover, a significant increase in GRβ protein expression in JEG-3 cells was seen after treatment with TGF-β1 (5 ng/mL) which corroborates previous research exhibiting TGF-β1 inhibitory action on GC signalling and hypothesised co-operation in the pathogenesis of GC resistance (302, 303, 306). These results do however contrast earlier studies demonstrating the upregulation of GR mRNA and protein expression in the U-937 monocyte cell line with evidence of optimised GC-GR binding after treatment with TGF-β1 (305). No significant alterations in GRα-C, GRP, GRA, nor GRα-D1-3 isotype protein expression were observed following treatments with TGF-β1 or SB431542. It is important to note that given the remarkable plasticity and context-dependent nature of both TGF-β1 transactivation and the GR, future research should be concerned with specific SMAD signal transducers and their effects on GR expression to better elucidate the underlying mechanisms in placental cell models.

The observed changes in GR isotype protein expression after hydrocortisone, TGF-β1, and combined treatments have seldom been explored in placental cells. These findings provide evidence of the potential roles altered GR transactivity as a consequence of perturbed GC and TGF-β1 signalling, as observed in cases of abnormal stress and inflammatory physiological conditions, may have in trophoblast dysfunction and associated pregnancy-related pathologies such as PE, IUGR, and PTB.

3.5.2 Protein expression of SERT using Western blot analysis

The present study investigated the effects of hydrocortisone and TGF-β1 on SERT protein expression in placental cell models for the first time. The banding pattern observed in the Western blot analysis for SERT protein consisted of several bands between weights of 55-200 kDa and these findings are supported by previous Western blot studies (331, 348-351). Within this range, two major bands were observed at ~50 kDa and ~68 kDa. Putative full-size SERT protein is widely recognised as being ~68 kDa and these bands were quantitated for the present study (330, 331). The banding at ~55 kDa, however, is a contentious issue in the literature, having been credited as non-glycosylated fragments of SERT or even being observed in SERT knock-out studies (228, 330, 331). Nevertheless, the prevailing theory behind the variation in SERT protein banding between weights of 55-200 kDa is considered to be due to fragmentation or aggregation, as well as protein modifications such as glycosylation and phosphorylation, among others (229, 232, 352, 353). Due to the challenges associated with unambiguous SERT protein detection, it is crucial to utilise positive and negative controls where possible to prevent erroneous measurements and subsequent conclusions. In the present study, A549 cells were once again used as both internal standards and positive controls after having been shown to express SERT (354). It is also worth noting that the SERT antibody (Thermo Fisher Scientific, Waltham, MA, USA) utilised in the present study had not been previously characterised in peer-reviewed journals.

Cortisol excess, represented by the hydrocortisone (1 μM) treatment group, demonstrated statistically significant increases in SERT protein expression which were reversed by mifepristone (10 nM) in both SWAN-71 and JEG-3 cell lines compared to vehicle controls (0.01% ethanol w/v). These findings corroborate previous reports of

GC-induced SERT expression in lymphocytes, neuronal cells, B-lymphoblastoid cells, murine brain tissue, and embryonic stem cell derived serotonergic neurons (269-273). As there were no observed changes in SERT expression between vehicle and untreated controls, and the effects were reversed by GR antagonist mifepristone, the present study can definitively claim that excess levels of hydrocortisone upregulate SERT protein expression in SWAN-71 and JEG-3 cells. In contrast, hydrocortisone (500 nM) and mifepristone (10 nM), individually or in combination, failed to alter SERT expression in both cell lines. These results are significant in that perturbed GC and serotonin signalling are frequently associated with placentation and pregnancy complications alike. It is worth noting that while immunoblotting indeed provides a semi-quantitative indication of altered SERT expression due to excess hydrocortisone, the underlying mechanisms remain unclear and various hypotheses persist. For instance, one possibility is that GC regulation of SERT expression is modulated by differential serotonin uptake by deletion/insertion genotypes of the SERT gene-promoter-linked polymorphic region (5-HTTLPR) in humans (271, 272). GCs may also regulate SERT expression through interactions with miR-16. Literature has previously indicated that SERT expression is regulated by miR-16 in the human placental cell line, JAR, where increased miR-16 post-transcriptionally downregulates SERT protein expression (277). Interestingly, low expression of miR-16 has also been associated with abnormal fetal growth (292). However, GCs have been shown to target and decrease miR-16 expression in various cell types which is contrary to the excess GCs and low miR-16 observed in trophoblast pathophysiological settings (355-359). Although ubiquitously expressed, it remains to be seen if miR-16 regulates SERT protein expression in trophoblasts. If that is indeed the case, this information suggests that GC-mediated upregulation of miR-16 and subsequent silencing of SERT expression is overwhelmed via 5-HTTLPR modulation

or other mechanisms yet to be elucidated. There also remains a possibility that GCs affect miR-16 in a context-dependent manner and, in contrast, induce downregulation or have no effect – as observed in dexamethasone-treated primary neurons *in vitro* (360). There may also be alternate responses in miR-16 expression as a result of acute or chronic excess glucocorticoid as miR-16 has been reported to be significantly downregulated in glucocorticoid-resistant patients with ulcerative colitis (361). Eventually, miR-16 physiology is complex and the effects of GC concentrations on miR-16 expression in placental cell models certainly warrants further investigation.

TGF-β1 (5 ng/mL) similarly increased SERT protein expression in SWAN-71 and JEG-3 cells as well as in combination with hydrocortisone (1 µM). These observations corroborate previous reports of TGF-β1-mediated upregulation of SERT protein in epithelial colorectal adenocarcinoma cell lines; however, this is the first time these interactions have been demonstrated in placental cells (295, 296). Treatment with the SMAD2/3 antagonist, SB431542 (1 µM), for 24 hours had no effect on SERT protein expression but did reverse the observed increase in SERT protein expression in the TGF-β1 treatment group in SWAN-71 cells. It also partly reversed the observed change in JEG-3 cells compared to both TGF-β1 vehicle control and DMSO vehicle control which definitively indicates TGF-β1 pharmacologic causal action on SERT. Moreover, TGF-β1 (5 ng/mL) and hydrocortisone (1 µM) combined treatment did not appear to yield an additive increase in SERT protein expression in the present study. It is known that TGF-β1 is upregulated throughout the course of pregnancy and plays an integral role in placentation and fetal development, which means that the present results provide a link to altered serotonin activity (200-205). SERT regulation by TGF-β1 has been attributed to PI3K, SMAD3, and STX3 mechanisms in previous research but require confirmation in placental cell models (295, 296).

3.5.3 mRNA expression of GR using NanoString nCounter analysis

The present study investigated total GR mRNA expression in the placental cell line, SWAN-71, after treatment with hydrocortisone, TGF-β1, or SB431542 for 24 hours using NanoString nCounter analysis. There were no statistically significant alterations in total GR mRNA expression after 24-hour treatment with hydrocortisone (1 μM), TGF-β1 (5 ng/mL), or SB431542 (1 μM). Research has shown that GR mRNA abundance is altered by treatment with GCs in both cultured animal and human cells. More specifically, the observed temporal reduction in GR mRNA expression in previous studies exhibits dose- and time-dependence with prolonged exposure to excess GCs leading to permanent changes in GR signalling (92, 93). The present study treated SWAN-71 cells with 1 μM hydrocortisone for 24 hours and, although a high concentration, did not significantly alter GR mRNA expression which suggests exposure time was too short and would subsequently account for the contrast with the research literature. Further, differences in individual GR isotype mRNA expression have been demonstrated following treatment with GCs and it may be the case that, because the present study measured total GR mRNA expression, these specific changes in GR isotype expression are poorly represented and subsequently undetected (362). Treatment of SWAN-71 cells with TGF-β1 (5 ng/mL) for 24 hours also had no effect on total GR mRNA expression which is in contrast with a previous report of TGF-β1-induced *de novo* GR synthesis showing increases in both GR protein expression and the steady-state level of GR mRNA (305). SMAD2/3 antagonist, SB431542, similarly failed to elicit a change in total GR mRNA expression. The research literature has

dismissed a role for post-transcriptional mechanisms and indicated that the underlying mechanisms could be transcriptional (305, 345). For example, SMAD2/3 may participate in the control of GR gene transcription by either interacting with the SMAD-binding element, complexing with AP-1 and interacting with the AP-1 site on the GR gene promoter, interfering with the transcription of genes encoding GR proteins, or even increasing the GR binding capacity of GR through the C-terminal activation domain of SMAD3 (305, 363, 364). Further, previous research has demonstrated that AP-1 might play a critical role in TGF-β1-induced GR regulation via putative upstream response elements (305). While it has also been shown that TGF-β1 may exert anti-inflammatory activities in part by increasing binding between GCs and GR, these effects were observed in the monocyte U-937 cell line with TGF-β1 concentrations of 10 ng/mL – twice the amount used in the present study – with evidence of a dose-dependent relationship (305). These findings suggest that the TGF-β1 (5 ng/mL) and SB431542 (1 μM) concentrations used in the present study may have been insufficient to elicit alterations in GR mRNA expression. Once again, it is important to note that the cross-regulation between the GR and TGF-β1 signalling pathways may be cell-specific and future research should be concerned with specific SMAD signal transducers and their effects on the mRNA expression of specific GR isotypes to better elucidate the underlying mechanisms in placental cell models.

These findings are significant to advancing contemporary understanding of the effects stress and inflammatory mediators, cortisol and TGF-β1, have on GR isotype and SERT protein expression as well as GR mRNA expression in trophoblast pathophysiology and their involvement in the aetiologies of PE, IUGR, and PTB.

CHAPTER 4: Overall discussion

Pregnancy-related conditions, PE, IUGR, and PTB, as well as maternal complications associated with gestation, such as GDM and PD, contribute significantly to both maternal and fetal morbidity and mortality making them a significant challenge in obstetric practice (1, 2, 12-17, 19). Although stress, inflammation, and the serotonin system have been implicated in each of these pathologies, their relationships in the placenta have seldom been investigated. More specifically, perturbations in the glucocorticoid, cortisol, the pro-inflammatory mediator, TGF-β1, and the serotonin transporter of the serotonin system have been associated with trophoblast dysfunction (19, 140-144, 157-159, 162, 201, 209-217). If abnormal cortisol, TGF-β1, and serotonin transporter signalling, as observed in cases of maternal stress, GDM, and PD, can lead to trophoblast dysfunction or inadequate placentation that results in the manifestation of PE, IUGR, or PTB, it indicates that these immune-endocrine interactions ultimately modulate various responses to environmental perturbations that may occur during gestation. These interactions, however, remain largely undefined. It was therefore the aim of the present study to investigate the potential interplay between GC, TGF-β, and serotonin system mechanisms in trophoblasts to refine our current understanding of their involvement in the aetiology of pregnancy-related pathologies. The premise was that subsequent findings will give rise to more sophisticated research that may ultimately lead to diagnostic, treatment, or preventative advances with a potential to influence obstetric practice.

SWAN-71 and JEG-3 placental cell lines were selected as *in vitro* models to investigate the effects of stress and inflammatory mediators, cortisol and TGF-β1, on trophoblast function and treated over a period of 24 hours with synthetic GC, hydrocortisone, at

100, 200, 500 nM, and 1 µM concentrations in the presence or absence of the potent GC antagonist, mifepristone, at 1, 5, and 10 nM concentrations. Cells were also treated with 5 ng/mL TGF-β1 in the presence or absence of 1 µM SMAD2/3 antagonist, SB431542. It was firstly paramount to elucidate the effects of these pharmacological agents on cell viability. This was determined through the utilisation of MTT and LDH assays. MTT measures cell metabolic activity while LDH measures cell cytotoxicity and, when taken in unison, these assays can provide a meaningful indication of total cell viability. The MTT assay indicated that there was a significant reduction in the metabolic activity of both SWAN-71 and JEG-3 cell lines following hydrocortisone and mifepristone treatments, which is met with mixed findings in recent research literature with evidence of both decreased and increased cell metabolism after treatment with GCs (310, 311). It is worth noting that an appreciable amount of prior research has included treatment with dexamethasone, a more potent glucocorticoid, with findings indicating cell type-specific MTT measurements with unequivocal discrepancies between hydrocortisone and dexamethasone with respect to cellular responses in general (311, 312). An interesting result was observed following combined hydrocortisone and mifepristone treatments in both SWAN-71 and JEG-3 cells where there was no synergetic nor inhibitory effect after each agent respectively decreased formazan production in the MTT assays. This might be due to the glucocorticoid agonist effects of mifepristone which have been reported in particular cell types and growth conditions independent to dose-response relationships (320). These agonist properties of mifepristone are not fully understood and warrant further investigation.

While these findings would, in itself, provide reasons to believe that cell viability might be compromised, results from the LDH assay provided evidence to the contrary. Following hydrocortisone and mifepristone treatments for 24 hours, there was no

observed increase in LDH which indicated the absence of a cytotoxic effect. Moreover, there were no significant differences observed in either MTT or LDH assays after TGF-β1 or SB431542 treatments relative to respective vehicle controls, and these findings contrast previous observations in the research literature of TGF-β1-induced increases in cell metabolism in JEG-3 cells after 6 hours (313). It should be noted, however, that the same study (313) noted gradual decreases in JEG-3 cell metabolic activity after 12 and 24 hours compared to untreated controls which appears to correlate with the results of the present study. The results from the cell viability assays revealed that although hydrocortisone and mifepristone altered both SWAN-71 and JEG-3 cell metabolic activity, the pharmacological compounds had no effect on cell cytotoxicity. The implications of this finding is that SWAN-71 and JEG-3 cell lines could be treated with hydrocortisone, mifepristone, TGF-β1, and SB431542 and subsequently utilised as *in vitro* models for investigating the effects of stress and inflammatory mediators on trophoblast physiology.

After cell viability assays were conducted, it was deemed appropriate that 500 nM and 1 μM hydrocortisone, 10 nM mifepristone, 5 ng/mL TGF-β1, and 1 μM SB431542 would comprise the pharmacological treatments for subsequent investigations on GR isotype and SERT protein and total GR mRNA expression. Western blot analysis was carried out with whole-cell lysates from treated cells where GR isoform protein expression was quantitated. It was demonstrated that treatment with hydrocortisone (1 μM) significantly reduced GRα-A expression in both SWAN-71 and JEG-3 cell lines after 24 hours which indicated that both cell lines were capable of GC resistance. These findings were significant as most gene expression and subsequent GR-mediated agonistic responses occur via the GRα-A isoform (83, 336). GR isotypes, GRβ, GRγ, GRP, GRA, GRα-C, and GRα-D1-3 protein expression were not affected by treatment with hydrocortisone

(500 nM or 1 μM) nor mifepristone (10 nM) after 24 hours in both cell lines. It is also worth noting that GRγ was not identified in the SWAN-71 cell line. Previous research has indicated that a decrease in GRα-A protein expression may be a result of upregulation of the dominant antagonist GRβ or a decrease in GRP, although neither of these responses were seen at protein level in the present study (85, 338, 341). The observed reductions in GRα-A protein expression appear to correlate with the decreased cell metabolic activity data from the MTT assays for both SWAN-71 and JEG-3 cell lines. For instance, the GRα-A isotype is a predominant target of GCs and may mediate their potent anti-proliferative effects (328). This was most evident in the SWAN-71 cell line in which excess hydrocortisone (1 μM) appeared to attenuate the decreased cell metabolic activity observed in lower concentrations of hydrocortisone (500, 200, and 100 nM) and this response might be due to GC resistance via reduced GRα-A protein expression. This was not observed in the JEG-3 cell line to a statistically significant extent. The underlying hypothetical mechanisms by which GCs regulate GR isotype expression warrant further investigation such as ligand-induced downregulation at the level of GR protein or GR mRNA including decreased mRNA stability, repressed GR promoter activity, or proteasomal degradation (85, 342-347).

TGF-β1 (5 ng/mL) similarly reduced GRα-A protein expression in both the SWAN-71 and JEG-3 cell lines after treatment for 24 hours and these findings appear to corroborate previous research, albeit in A549 cells (304). A significant increase in GRβ was also seen after 24-hour treatment with TGF-β1, which correlated with some but not all research on different cell types in published literature (302, 303, 305, 306). Additionally, treatment with TGF-β1 and hydrocortisone (1 μM), in combination, similarly decreased GRα-A with no additive effect. Moreover, there were no changes in the remaining GR isotypes following TGF-β1 nor SB431542 treatments after 24 hours.

It should be noted that although it has been observed that TGF-β1 alters GRα-A and GRβ protein expression the underlying mechanisms remain poorly understood and future studies ought to investigate the roles of SMAD signal transducers or AP-1 and the manner in which they interact with promoters, GR binding capacity, or the GR-encoding genes themselves (305, 363, 364). The findings of the present study are novel in placental cell models and provide evidence of stress- and inflammatory-induced perturbations in GR physiology that would hypothetically modify the expression of a multitude of placental genes crucial for placentation and sustaining fetal life (85).

SERT protein expression was also quantitated through Western blot analysis where hydrocortisone (1 μM) was demonstrated to increase SERT protein expression in both SWAN-71 and JEG-3 cell lines to a statistically significant extent. The research literature indicates that GCs may upregulate SERT in various ways, including GC modulation of the 5-HTTLPR or the GC-mediated regulation of miR-16, and these hypotheses warrant further investigation (271, 272, 360, 361). Further, the present study demonstrates for the first time that 24-hour treatment with TGF-β1 (5 ng/mL), in isolation and in combination with hydrocortisone (1 μM), similarly increases SERT protein expression in both SWAN-71 and JEG-3 cells. SB431542 (1 μM) had no effect on SERT protein expression. SERT regulation by TGF-β1 has been attributed to PI3K, SMAD3, and STX3 mechanisms in previous studies but this has not been investigated in placental cell models (295, 296). Serotonin plays a key role in early embryogenesis and enhances trophoblast viability and progression through the cell cycle so that the observed upregulation of SERT protein may impede crucial serotonin system signalling and have deleterious effects on trophoblast function (238, 239). It is possible that, in a manner similar to the GC- and TGF-β1-induced downregulation of GRα-A and subsequent attenuation of the GC anti-proliferative effect observed in SWAN-71 cells,

the GC- and TGF-β1-induced upregulation of SERT may mediate serotonin system proliferative effects observed in JEG-3 cells although, without further investigations, these assertions remain only speculative.

Finally, NanoString nCounter was carried out with whole-cell nucleic acid samples from treated SWAN-71 cells where total GR mRNA were quantitated. It was demonstrated that neither hydrocortisone (1 μM) nor TGF-β1 (5 ng/mL) altered total GR mRNA expression to a statistically significant extent. Taken together with the GR protein expression data, the results of the present study suggest that the observed alterations in GRα-A or GRβ might occur at the posttranscriptional and/or translational level. However, as only total GR mRNA expression was measured rather than the mRNA expression of specific GR isotypes, it cannot be asserted that there were no changes in GR isotype mRNA expression. Additionally, future investigations should aim to elucidate the roles of SMAD3 and AP-1 and their interactions with promoters, GR binding capacity, or the GR-encoding genes, which have been hypothesised in the research literature, to gain insight into the underlying mechanisms involved (305, 363, 364).

There were several limitations in the present study design that affected the magnitude and significance of the findings. One of the most pressing limitations pertains to the FBS supplementation of the cell culture media which may have contained traces of cortisol. This meant that cells may have been systematically pre-treated with cortisol prior to and during pharmacological treatments which may have had profound effects on protein and mRNA expression results after treatment with hydrocortisone. It is therefore recommended that future investigations quantitate transient pharmacological agents present in FBS-supplemented media. With respect to the cell viability studies, the MTT and LDH assays respectively assessed cell metabolic activity and cytotoxicity which,

taken together, provide a meaningful indication of overall cell viability. Another test, such as an image-based analysis, to directly measure cell proliferation would provide additional and accurate characterisation of the cell type's response to the pharmacological compounds. Next, the GR isotype protein expression results lacked explanatory power with respect to the absolute physiological processes taking place; because whole-cell lysates were analysed through Western blotting, there was no indication of whether the specific GR isotype proteins were in the cytoplasm awaiting ligand binding or in the nucleus changing genes, which is a pertinent concern for GR physiology. Future studies should therefore consider cell fractionation assessments of GR isotype protein expression. Further, although statistically significant increases in SERT protein expression were elucidated after treatments, once again, the whole-cell lysates analysed in the present study via Western blotting scarcely explain the absolute physiological processes taking place. For instance, a total increase in the cellular abundance of SERT does not mean that there is an increase in SERT expression on the plasma membrane or that SERT exists in a conformation that allows serotonin binding. SERT uptake functionality depends on its structure, folding, membrane trafficking, association with other proteins, and assembly on the plasma membrane where contemporary theories suggest that these activities are regulated by post-translational modifications which are seldom explored in the present study (228-231). Future investigations should consider subcellular fractionation studies that include plasma membrane fractions and seek to identify glycosylated, phosphorylated, and oligomerized SERT proteins. Another limitation for the quantitation of SERT protein via Western blot analysis was that the SERT antibody utilised in the present study had not been previously characterised in peer-reviewed journals which, along with discrepancies persisting in contemporary literature with respect to SERT western blot

analysis, suggests that the banding measured at ~68 kDa may be an unreliable marker (331). Additionally, the NanoString nCounter analysis of total GR expression should have included combined antagonist treatments to assess whether any observed changes in mRNA expression were directly caused by agonist action. Specific GR isotypes and SERT mRNA should have also been included in analysis to allow for meaningful comparisons to protein expression studies. Moreover, Western blot densitometric quantitation of proteins is only a semi-quantitative measure so results ought to be verified with enzyme-linked immunosorbent assay (ELISA) or future studies may quantitate protein expression through mass spectrometry to avoid using antibodies. The present study investigated the effects of stress and inflammatory mediators on protein and mRNA expression in cell lines, SWAN-71 and JEG-3, which, although remarkably similar to *in* vivo trophoblast cells, lack the specific cell-cell interactions and it is known that cell lines may undergo genetic drift (365). As such, a progression to placental extracts, primary trophoblast cells with co-culture measures, or *in vivo* experiments should be considered for future investigations to optimise the generalisability of findings.

Overall, the present study demonstrated that both the SWAN-71 and JEG-3 cell lines are suitable placental cell models for investigations pertaining to stress and inflammation and subsequent effects on trophoblast physiology. Stress- and inflammatory-induced alterations in GR and SERT protein expression through the direct action of hydrocortisone and TGF-β1 were also shown. While the specific underlying mechanisms of these cellular responses warrant further investigation, it can be deduced that perturbations in cortisol and TGF-β1 processes can have pronounced effects on trophoblast function with respect to GC and serotonin system signalling and may play key roles in the aetiologies of pregnancy-related pathologies such as PE, IUGR, and

PTB. These findings allow for more directed research to follow that may ultimately lead to diagnostic, treatment, or preventative advances in the future to attenuate the maternal and fetal morbidity and mortality in clinical practice.

References

1. Sava RI, March KL, Pepine CJ. Hypertension in pregnancy: Taking cues from pathophysiology for clinical practice. Clin Cardiol. 2018;41(2):220-7.
2. Chaiworapongsa T, Chaemsaithong P, Yeo L, Romero R. Pre-eclampsia part 1: current understanding of its pathophysiology. Nature Reviews Nephrology. 2014;10(8):466.
3. Inoue H, Nishio H, Takada H, Sakai Y, Nanishi E, Ochiai M, et al. Activation of nod1 signaling induces fetal growth restriction and death through fetal and maternal vasculopathy. The Journal of Immunology. 2016:1500295.
4. Suhag A, Berghella V. Intrauterine growth restriction (IUGR): etiology and diagnosis. Current Obstetrics and Gynecology Reports. 2013;2(2):102-11.
5. von Beckerath A-K, Kollmann M, Rotky-Fast C, Karpf E, Lang U, Klaritsch P. Perinatal complications and long-term neurodevelopmental outcome of infants with intrauterine growth restriction. American Journal of Obstetrics and Gynecology. 2013;208(2):130. e1-. e6.
6. Bernstein IM, Horbar JD, Badger GJ, Ohlsson A, Golan A, Network VO. Morbidity and mortality among very-low-birth-weight neonates with intrauterine growth restriction. American Journal of Obstetrics and Gynecology. 2000;182(1):198-206.
7. Tolsa CB, Zimine S, Warfield SK, Freschi M, Rossignol AS, Lazeyras F, et al. Early alteration of structural and functional brain development in premature infants born with intrauterine growth restriction. Pediatric Research. 2004;56(1):132.
8. Devaskar SU, Chu A. Intrauterine growth restriction: hungry for an answer. Physiology. 2016;31(2):131-46.

9. Longo S, Bollani L, Decembrino L, Di Comite A, Angelini M, Stronati M. Short-term and long-term sequelae in intrauterine growth retardation (IUGR). The Journal of Maternal-Fetal & Neonatal Medicine. 2013;26(3):222-5.
10. McGrath M, Sullivan M. Birth weight, neonatal morbidities, and school age outcomes in full-term and preterm infants. Comprehensive Child and Adolescent Nursing. 2002;25(4):231-54.
11. Blencowe H, Lee AC, Cousens S, Bahalim A, Narwal R, Zhong N, et al. Preterm birth-associated neurodevelopmental impairment estimates at regional and global levels for 2010. Pediatric Research. 2013;74 Suppl 1:17-34.
12. Blencowe H, Cousens S, Chou D, Oestergaard M, Say L, Moller AB, et al. Born too soon: the global epidemiology of 15 million preterm births. Reproductive Health. 2013;10 Suppl 1:S2.
13. Liu L, Oza S, Hogan D, Perin J, Rudan I, Lawn JE, et al. Global, regional, and national causes of child mortality in 2000-13, with projections to inform post-2015 priorities: an updated systematic analysis. Lancet. 2015;385(9966):430-40.
14. Oza S, Lawn JE, Hogan DR, Mathers C, Cousens SN. Neonatal cause-of-death estimates for the early and late neonatal periods for 194 countries: 2000-2013. Bulletin of the World Health Organization. 2015;93(1):19-28.
15. Melchior H, Kurch-Bek D, Mund M. The Prevalence of Gestational Diabetes. Deutsches Ärzteblatt international. 2017;114(24):412-8.
16. Lekva T, Godang K, Michelsen AE, Qvigstad E, Normann KR, Norwitz ER, et al. Prediction of Gestational Diabetes Mellitus and Pre-diabetes 5 Years Postpartum using 75 g Oral Glucose Tolerance Test at 14–16 Weeks' Gestation. Scientific Reports. 2018;8(1):13392.

17. Ferrara A. Increasing prevalence of gestational diabetes mellitus: a public health perspective. Diabetes Care. 2007;30 Suppl 2:S141-6.

18. Viau M, Lafond J, Vaillancourt C. Expression of placental serotonin transporter and 5-HT 2A receptor in normal and gestational diabetes mellitus pregnancies. Reproductive BioMedicine Online. 2009;19(2):207-15.

19. Brummelte S, Galea LA. Depression during pregnancy and postpartum: contribution of stress and ovarian hormones. Progress in Neuro-Psychopharmacology & Biological Psychiatry. 2010;34(5):766-76.

20. Eke AC, Saccone G, Berghella V. Selective serotonin reuptake inhibitor (SSRI) use during pregnancy and risk of preterm birth: a systematic review and meta-analysis. BJOG. 2016;123(12):1900-7.

21. Grote NK, Bridge JA, Gavin AR, Melville JL, Iyengar S, Katon WJ. A meta-analysis of depression during pregnancy and the risk of preterm birth, low birth weight, and intrauterine growth restriction. JAMA Psychiatry. 2010;67(10):1012-24.

22. Grote NK, Bridge JA, Gavin AR, Melville JL, Iyengar S, Katon WJ. A meta-analysis of depression during pregnancy and the risk of preterm birth, low birth weight, and intrauterine growth restriction. JAMA Psychiatry. 2010;67(10):1012-24.

23. Brummelte S, Schmidt KL, Taves MD, Soma KK, Galea LA. Elevated corticosterone levels in stomach milk, serum, and brain of male and female offspring after maternal corticosterone treatment in the rat. Developmental Neurobiology. 2010;70(10):714-25.

24. Leinonen JA, Solantaus TS, Punamaki RL. Parental mental health and children's adjustment: the quality of marital interaction and parenting as mediating factors. J Journal of Child Psychology and Psychiatry. 2003;44(2):227-41.

25. Bauer A, Pawlby S, Plant DT, King D, Pariante CM, Knapp M. Perinatal depression and child development: exploring the economic consequences from a South London cohort. Psychological Medicine. 2015;45(1):51-61.

26. Khan KA, Petrou S, Dritsaki M, Johnson SJ, Manktelow B, Draper ES, et al. Economic costs associated with moderate and late preterm birth: a prospective population-based study. BJOG: An International Journal of Obstetrics & Gynaecology. 2015;122(11):1495-505.

27. Li R, Tsigas EZ, Callaghan WM. Health and economic burden of preeclampsia: no time for complacency. American Journal of Obstetrics & Gynecology. 2017;217(3):235-6.

28. Marzouk A, Filipovic-Pierucci A, Baud O, Tsatsaris V, Ego A, Charles M-A, et al. Prenatal and post-natal cost of small for gestational age infants: a national study. BMC Health Services Research. 2017;17(1):221.

29. Meregaglia M, Dainelli L, Banks H, Benedetto C, Detzel P, Fattore G. The short-term economic burden of gestational diabetes mellitus in Italy. BMC Pregnancy and Childbirth. 2018;18(1):58.

30. Okae H, Toh H, Sato T, Hiura H, Takahashi S, Shirane K, et al. Derivation of human trophoblast stem cells. Cell Stem Cell. 2018;22(1):50-63. e6.

31. James JL, Carter AM, Chamley LW. Human placentation from nidation to 5 weeks of gestation. Part I: What do we know about formative placental development following implantation? Placenta. 2012;33(5):327-34.

32. Rossant J, Cross JC. Placental development: lessons from mouse mutants. Nature Reviews Genetics. 2001;2(7):538.

33. Nandi P, Lim H, Torres-Garcia EJ, Lala PK. Human trophoblast stem cell self-renewal and differentiation: Role of decorin. Scientific Reports. 2018;8(1):8977.

34. Burton G, Woods A, Jauniaux E, Kingdom J. Rheological and physiological consequences of conversion of the maternal spiral arteries for uteroplacental blood flow during human pregnancy. Placenta. 2009;30(6):473-82.

35. Soares MJ, Iqbal K, Kozai K. Hypoxia and placental development. Birth Defects Research. 2017;109(17):1309-29.

36. Sibai B, Dekker G, Kupferminc M. Pre-eclampsia. The Lancet. 2005;365(9461):785-99.

37. Pijnenborg R, Vercruysse L, Hanssens M. The uterine spiral arteries in human pregnancy: facts and controversies. Placenta. 2006;27(9-10):939-58.

38. Huppertz B, Gauster M, Orendi K, König J, Moser G. Oxygen as modulator of trophoblast invasion. Journal of Anatomy. 2009;215(1):14-20.

39. Ohmaru-Nakanishi T, Asanoma K, Fujikawa M, Fujita Y, Yagi H, Onoyama I, et al. Fibrosis in Preeclamptic Placentas Is Associated with Stromal Fibroblasts Activated by the Transforming Growth Factor-β1 Signaling Pathway. The American Journal of Pathology. 2018;188(3):683-95.

40. Zeisberg M, Kalluri R. Cellular mechanisms of tissue fibrosis. 1. Common and organ-specific mechanisms associated with tissue fibrosis. American Journal of Physiology-Cell Physiology. 2012;304(3):C216-C25.

41. Askelund K, Chamley L. Trophoblast deportation part I: review of the evidence demonstrating trophoblast shedding and deportation during human pregnancy. Placenta. 2011;32(10):716-23.

42. Johansen M, Redman C, Wilkins T, Sargent I. Trophoblast deportation in human pregnancy—its relevance for pre-eclampsia. Placenta. 1999;20(7):531-9.

43. Attwood H, Park W. Embolism to the lungs by trophoblast. BJOG: An International Journal of Obstetrics & Gynaecology. 1961;68(4):611-7.

44. Jäämeri K, Koivuniemi A, Carpen E. Occurrence of trophoblasts in the blood of toxaemic patients. Gynecologic and Obstetric Investigation. 1965;160(5):315-20.

45. Pantham P, Askelund K, Chamley L. Trophoblast deportation part II: a review of the maternal consequences of trophoblast deportation. Placenta. 2011;32(10):724-31.

46. Frøen JF, Gardosi JO, Thurmann A, Francis A, Stray-Pedersen B. Restricted fetal growth in sudden intrauterine unexplained death. Acta Obstetricia et Gynecologica Scandinavica. 2004;83(9):801-7.

47. Saleem T, Sajjad N, Fatima S, Habib N, Ali SR, Qadir M. Intrauterine growth retardation-small events, big consequences. Italian Journal of Pediatrics. 2011;37(1):41.

48. Wilkins-Haug L, Roberts D, Morton C. Confined placental mosaicism and intrauterine growth retardation: a case-control analysis of placentas at delivery. American Journal of Obstetrics & Gynecology. 1995;172(1):44-50.

49. Sato Y, Benirschke K, Marutsuka K, Yano Y, Hatakeyama K, Iwakiri T, et al. Associations of intrauterine growth restriction with placental pathological factors, maternal factors and fetal factors; clinicopathological findings of 257 Japanese cases. Histology & Histopathology. 2013;28(1):127-32.

50. Smith SC, Baker PN, Symonds EM. Increased placental apoptosis in intrauterine growth restriction. American Journal of Obstetrics and Gynecology. 1997;177(6):1395-401.

51. Morgan TK. Role of the placenta in preterm birth: a review. American journal of perinatology. 2016;33(03):258-66.
52. Moll W. Structure adaptation and blood flow control in the uterine arterial system after hemochorial placentation. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2003;110:S19-S27.
53. James JL, Cartwright JE, Whitley GS, Greenhill DR, Hoppe A. The regulation of trophoblast migration across endothelial cells by low shear stress: consequences for vascular remodelling in pregnancy. Cardiovascular research. 2011;93(1):152-61.
54. Ohara-Imaizumi M, Kim H, Yoshida M, Fujiwara T, Aoyagi K, Toyofuku Y, et al. Serotonin regulates glucose-stimulated insulin secretion from pancreatic beta cells during pregnancy. Proc Natl Acad Sci U S A. 2013;110(48):19420-5.
55. Green IC, Taylor KW. Effects of pregnancy in the rat on the size and insulin secretory response of the islets of Langerhans. J Endocrinol. 1972;54(2):317-25.
56. Sorenson RL, Brelje TC. Adaptation of islets of Langerhans to pregnancy: beta-cell growth, enhanced insulin secretion and the role of lactogenic hormones. Horm Metab Res. 1997;29(6):301-7.
57. Rieck S, Kaestner KH. Expansion of beta-cell mass in response to pregnancy. Trends Endocrinol Metab. 2010;21(3):151-8.
58. Buchanan TA, Xiang AH. Gestational diabetes mellitus. J Clin Invest. 2005;115(3):485-91.
59. Kim C, Newton KM, Knopp RH. Gestational diabetes and the incidence of type 2 diabetes: a systematic review. Diabetes care. 2002;25(10):1862-8.

60. Desoye G, Hauguel-de Mouzon S. The human placenta in gestational diabetes mellitus: the insulin and cytokine network. Diabetes Care. 2007;30(Supplement 2):S120-S6.

61. Miyakoshi K, Tanaka M, Matsumoto T, Hattori Y, Ueno K, Teranishi T, et al. Hypertensive disorders in Japanese women with gestational glucose intolerance. Diabetes Research and Clinical Practice. 2004;64(3):201-5.

62. Lee J, Ouh Y-t, Ahn KH, Hong SC, Oh M-J, Kim H-J, et al. Preeclampsia: A risk factor for gestational diabetes mellitus in subsequent pregnancy. PloS One. 2017;12(5):e0178150.

63. Bryson CL, Ioannou GN, Rulyak SJ, Critchlow C. Association between gestational diabetes and pregnancy-induced hypertension. American Journal of Epidemiology. 2003;158(12):1148-53.

64. Talge NM, Neal C, Glover V, Early Stress TR, Fetal PSN, Child NEo, et al. Antenatal maternal stress and long-term effects on child neurodevelopment: how and why? Journal of Child Psychology and Psychiatry. 2007;48(3-4):245-61.

65. Van den Bergh BR, Van Calster B, Smits T, Van Huffel S, Lagae L. Antenatal maternal anxiety is related to HPA-axis dysregulation and self-reported depressive symptoms in adolescence: a prospective study on the fetal origins of depressed mood. Neuropsychopharmacology. 2008;33(3):536.

66. Sandman CA, Buss C, Head K, Davis EP. Fetal exposure to maternal depressive symptoms is associated with cortical thickness in late childhood. Biological Psychiatry. 2015;77(4):324-34.

67. Plant D, Pawlby S, Sharp D, Zunszain P, Pariante C. Prenatal maternal depression is associated with offspring inflammation at 25 years: a prospective longitudinal cohort study. Translational Psychiatry. 2016;6(11):e936.

68. Pariante CM, Lightman SL. The HPA axis in major depression: classical theories and new developments. Trends in Neurosciences. 2008;31(9):464-8.

69. Raison CL, Capuron L, Miller AH. Cytokines sing the blues: inflammation and the pathogenesis of depression. Trends in Immunology. 2006;27(1):24-31.

70. Horowitz M, Zunszain P, Anacker C, Musaelyan K, Pariante C. Glucocorticoids and Inflammation: A Double-Headed Sword in Depression? Inflammation in Psychiatry. 28: Karger Publishers; 2013. p. 127-43.

71. Smith SM, Vale WW. The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. Dialogues in Clinical Neuroscience. 2006;8(4):383.

72. Carrasco GA, Van de Kar LD. Neuroendocrine pharmacology of stress. European Journal of Pharmacology. 2003;463(1-3):235-72.

73. Stephens MAC, Wand G. Stress and the HPA axi: Role of glucocorticoids in alcohol dependence. Alcohol Research: Rurrent Reviews. 2012.

74. Charmandari E, Tsigos C, Chrousos G. Endocrinology of the stress response. Annual Review of Physiology. 2005;67:259-84.

75. Joseph D, Whirledge S. Stress and the hpa axis: Balancing homeostasis and fertility. International Journal of Molecular Sciences. 2017;18(10):2224.

76. Schmid W, Cole T, Blendy J, Schütz G. Molecular genetic analysis of glucocorticoid signalling in development. The Journal of steroid biochemistry and molecular biology. 1995;53(1-6):33-5.

77. Grad I, Picard D. The glucocorticoid responses are shaped by molecular chaperones. Molecular and Cellular Endocrinology. 2007;275(1-2):2-12.

78. Weikum ER, Knuesel MT, Ortlund EA, Yamamoto KR. Glucocorticoid receptor control of transcription: precision and plasticity via allostery. Nature Reviews Molecular Cell Biology. 2017;18(3):159-74.

79. Weikum ER, Knuesel MT, Ortlund EA, Yamamoto KR. Glucocorticoid receptor control of transcription: precision and plasticity via allostery. Nature Reviews Molecular Cell Biology. 2017;18(3):159.

80. Pratt WB, Toft DO. Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocrine Reviews. 1997;18(3):306-60.

81. Luisi BF, Xu W, Otwinowski Z, Freedman L, Yamamoto K, Sigler P. Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature. 1991;352(6335):497.

82. Lu NZ, Cidlowski JA. Translational regulatory mechanisms generate N-terminal glucocorticoid receptor isoforms with unique transcriptional target genes. Molecular Cell. 2005;18(3):331-42.

83. Lu NZ, Collins JB, Grissom SF, Cidlowski JA. Selective regulation of bone cell apoptosis by translational isoforms of the glucocorticoid receptor. Molecular and Cellular Biology. 2007;27(20):7143-60.

84. Turner JD, Schote AB, Keipes M, Muller CP. A new transcript splice variant of the human glucocorticoid receptor. Annals of the New York Academy of Sciences. 2007;1095(1):334-41.

85. Bivol S, Owen SJ, Rose'Meyer RB. Glucocorticoid-induced changes in glucocorticoid receptor mRNA and protein expression in the human placenta as a potential factor for altering fetal growth and development. Reproduction, Fertility and Development. 2017;29(5):845-54.

86. Lewis-Tuffin LJ, Jewell CM, Bienstock RJ, Collins JB, Cidlowski JA. Human glucocorticoid receptor β binds RU-486 and is transcriptionally active. Molecular and Cellular Biology. 2007;27(6):2266-82.

87. Pujols L, Mullol J, Roca-Ferrer J, Torrego A, Xaubet A, Cidlowski JA, et al. Expression of glucocorticoid receptor α-and β-isoforms in human cells and tissues. American Journal of Physiology-Cell Physiology. 2002;283(4):C1324-C31.

88. Oakley RH, Webster JC, Sar M, Parker Jr CR, Cidlowski JA. Expression and subcellular distribution of the β-isoform of the human glucocorticoid receptor. Endocrinology. 1997;138(11):5028-38.

89. Oakley RH, Jewell CM, Yudt MR, Bofetiado DM, Cidlowski JA. The dominant negative activity of the human glucocorticoid receptor β isoform specificity and mechanisms of action. Journal of Biological Chemistry. 1999;274(39):27857-66.

90. Oakley RH, Sar M, Cidlowski JA. The human glucocorticoid receptor isoform expression, biochemical properties, and putative function. Journal of Biological Chemistry. 1996;271(16):9550-9.

91. Kino T, Manoli I, Kelkar S, Wang Y, Su YA, Chrousos GP. Glucocorticoid receptor (GR) β has intrinsic, GRα-independent transcriptional activity. Biochemical and Biophysical Research Communications. 2009;381(4):671-5.

92. Cidlowski NB, Cidlowski JA. Regulation of glucocorticoid receptors by glucocorticoids in cultured HeLa S3 cells. Endocrinology. 1981;109(6):1975-82.

93. Silva CM, Powell-Oliver FE, Jewell CM, Sar M, Allgood VE, Cidlowski JA. Regulation of the human glucocorticoid receptor by long-term and chronic treatment with glucocorticoid. Steroids. 1994;59(7):436-42.

94. Liggins G. The role of cortisol in preparing the fetus for birth. Reproduction, Fertility and Development. 1994;6(2):141-50.

95. Seckl JR, Holmes MC. Mechanisms of disease: glucocorticoids, their placental metabolism and fetal 'programming' of adult pathophysiology. Nature Clinical Practice Endocrinology & Metabolism. 2007;3:479.

96. Akinloye O, Obikoya O, Jegede A, Oparinde D, Arowojolu A. Cortisol plays central role in biochemical changes during pregnancy. International Journal of Medicine and Biomedical Research. 2013;2(1):3-12.

97. Glynn LM, Sandman CA. Sex moderates associations between prenatal glucocorticoid exposure and human fetal neurological development. Developmental Science. 2012;15(5):601-10.

98. Kitraki E, Kittas C, Stylianopoulou F. Glucocorticoid receptor gene expression during rat embryogenesis. An in situ hybridization study. Differentiation. 1997;62(1):21-31.

99. Demey-Ponsart E, Foidart J-M, Sulon J, Sodoyez J. Serum CBG, free and total cortisol and circadian patterns of adrenal function in normal pregnancy. Journal of Steroid Biochemistry. 1982;16(2):165-9.

100. Lindsay JR, Nieman LK. The hypothalamic-pituitary-adrenal axis in pregnancy: challenges in disease detection and treatment. Endocrine Reviews. 2005;26(6):775-99.

101. Phocas I, Sarandakou A, Rizos D. Maternal serum total cortisol levels in normal and pathologic pregnancies. Int J Gynaecol Obstet. 1990;31(1):3-8.

102. Thomson M. The physiological roles of placental corticotropin releasing hormone in pregnancy and childbirth. Journal of Physiology and Biochemistry. 2013;69(3):559-73.

103. Stroupe S, Gray R, Westphal U. Steroid–protein interactions Kinetics of binding of cortisol and progesterone to human corticosteroid-binding globulin. FEBS Letters. 1978;86(1):61-4.
104. Blackburn S. The hypothalamic-pituitary-adrenal axis during pregnancy. The Journal of Perinatal & Neonatal Nursing. 2010;24(1):10-1.
105. Beitins IZ, Bayard F, Ances IG, Kowarski A, Migeon CJ. The metabolic clearance rate, blood production, interconversion and transplacental passage of cortisol and cortisone in pregnancy near term. Pediatric Research. 1973;7(5):509.
106. Chapman K, Holmes M, Seckl J. 11β-hydroxysteroid dehydrogenases: intracellular gate-keepers of tissue glucocorticoid action. Physiological Reviews. 2013;93(3):1139-206.
107. Dalle M, Giry J, Gay M, Delost P. Perinatal changes in plasma and adrenal corticosterone and aldosterone concentrations in the mouse. Journal of Endocrinology. 1978;76(2):303-9.
108. Montano MM, Wang M-H, Even MD, Vom Saal FS. Serum corticosterone in fetal mice: sex differences, circadian changes, and effect of maternal stress. Physiology & Behavior. 1991;50(2):323-9.
109. Gitau R, Cameron A, Fisk NM, Glover V. Fetal exposure to maternal cortisol. The Lancet. 1998;352(9129):707-8.
110. Brown R, Diaz R, Robson A, Kotelevtsev Y, Mullins J, Kaufman M, et al. The ontogeny of 11 beta-hydroxysteroid dehydrogenase type 2 and mineralocorticoid receptor gene expression reveal intricate control of glucocorticoid action in development. Endocrinology. 1996;137(2):794-7.

111. Brown RW, Chapman KE, Edwards C, Seckl JR. Human placental 11 beta-hydroxysteroid dehydrogenase: evidence for and partial purification of a distinct NAD-dependent isoform. Endocrinology. 1993;132(6):2614-21.
112. Krozowski Z, MaGuire JA, Stein-Oakley AN, Dowling J, Smith RE, Andrews RK. Immunohistochemical localization of the 11 beta-hydroxysteroid dehydrogenase type II enzyme in human kidney and placenta. The Journal of Clinical Endocrinology & Metabolism. 1995;80(7):2203-9.
113. Stewart PM, Rogerson FM, Mason J. Type 2 11 beta-hydroxysteroid dehydrogenase messenger ribonucleic acid and activity in human placenta and fetal membranes: its relationship to birth weight and putative role in fetal adrenal steroidogenesis. The Journal of Clinical Endocrinology & Metabolism. 1995;80(3):885-90.
114. Urizar Jr GG, Muñoz RF. Impact of a prenatal cognitive-behavioral stress management intervention on salivary cortisol levels in low-income mothers and their infants. Psychoneuroendocrinology. 2011;36(10):1480-94.
115. Allen LH. Biological mechanisms that might underlie iron's effects on fetal growth and preterm birth. The Journal of Nutrition. 2001;131(2):581S-9S.
116. Lesage J, Blondeau B, Grino M, Breant B, Dupouy J. Maternal undernutrition during late gestation induces fetal overexposure to glucocorticoids and intrauterine growth retardation, and disturbs the hypothalamo-pituitary adrenal axis in the newborn rat. Endocrinology. 2001;142(5):1692-702.
117. Field T, Diego M, Hernandez-Reif M. Prenatal depression effects and interventions: a review. Infant Behavior and Development. 2010;33(4):409-18.
118. Ford SP, Zhang L, Zhu M, Miller MM, Smith DT, Hess BW, et al. Maternal obesity accelerates fetal pancreatic β-cell but not α-cell development in sheep:

prenatal consequences. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2009;297(3):R835-R43.

119. Matthews S, Owen D, Banjanin S, Andrews M. Glucocorticoids, hypothalamo-pituitary-adrenal (HPA) development, and life after birth. Endocrine Research. 2002;28(4):709-18.

120. Hardy D, Yang K. The expression of 11β-hydroxysteroid dehydrogenase type 2 is induced during trophoblast differentiation: effects of hypoxia. The Journal of Clinical Endocrinology & Metabolism. 2002;87(8):3696-701.

121. O'Donnell KJ, Jensen AB, Freeman L, Khalife N, O'Connor TG, Glover V. Maternal prenatal anxiety and downregulation of placental 11β-HSD2. Psychoneuroendocrinology. 2012;37(6):818-26.

122. Saif Z, Hodyl N, Hobbs E, Tuck A, Butler M, Osei-Kumah A, et al. The human placenta expresses multiple glucocorticoid receptor isoforms that are altered by fetal sex, growth restriction and maternal asthma. Placenta. 2014;35(4):260-8.

123. Clifton V, Cuffe J, Moritz K, Cole T, Fuller P, Lu N, et al. The role of multiple placental glucocorticoid receptor isoforms in adapting to the maternal environment and regulating fetal growth. Placenta. 2017;54:24-9.

124. Saif Z, Hodyl N, Stark M, Fuller P, Cole T, Lu N, et al. Expression of eight glucocorticoid receptor isoforms in the human preterm placenta vary with fetal sex and birthweight. Placenta. 2015;36(7):723-30.

125. Cao-Lei L, Leija SC, Kumsta R, Wüst S, Meyer J, Turner JD, et al. Transcriptional control of the human glucocorticoid receptor: identification and analysis of alternative promoter regions. Human Genetics. 2011;129(5):533-43.

126. Stroud LR, Papandonatos GD, Rodriguez D, McCallum M, Salisbury AL, Phipps MG, et al. Maternal smoking during pregnancy and infant stress response: test of a prenatal programming hypothesis. Psychoneuroendocrinology. 2014;48:29-40.
127. Kertes DA, Kamin HS, Hughes DA, Rodney NC, Bhatt S, Mulligan CJ. Prenatal maternal stress predicts methylation of genes regulating the hypothalamic–pituitary–adrenocortical system in mothers and newborns in the Democratic Republic of Congo. Child Development. 2016;87(1):61-72.
128. Filiberto AC, Maccani MA, Koestler DC, Wilhelm-Benartzi C, Avissar-Whiting M, Banister CE, et al. Birthweight is associated with DNA promoter methylation of the glucocorticoid receptor in human placenta. Epigenetics. 2011;6(5):566-72.
129. Conradt E, Lester BM, Appleton AA, Armstrong DA, Marsit CJ. The roles of DNA methylation of NR3C1 and 11β-HSD2 and exposure to maternal mood disorder in utero on newborn neurobehavior. Epigenetics. 2013;8(12):1321-9.
130. Hagendorf A, Koper JW, de Jong FH, Brinkmann AO, Lamberts SW, Feelders RA. Expression of the human glucocorticoid receptor splice variants α, β, and P in peripheral blood mononuclear leukocytes in healthy controls and in patients with hyper-and hypocortisolism. The Journal of Clinical Endocrinology & Metabolism. 2005;90(11):6237-43.
131. Dorr HG, Heller A, Versmold HT, Sippell WG, Herrmann M, Bidlingmaier F, et al. Longitudinal study of progestins, mineralocorticoids, and glucocorticoids throughout human pregnancy. The Journal of Clinical Endocrinology and Metabolism. 1989;68(5):863-8.
132. Odagiri E, Ishiwatari N, Abe Y, Jibiki K, Adachi T, Demura R, et al. Hypercortisolism and the resistance to dexamethasone suppression during gestation. Endocrinology Japan. 1988;35(5):685-90.

133. Jung C, Ho JT, Torpy DJ, Rogers A, Doogue M, Lewis JG, et al. A longitudinal study of plasma and urinary cortisol in pregnancy and postpartum. The Journal of Clinical Endocrinology & Metabolism. 2011;96(5):1533-40.
134. Mastorakos G, Ilias I. Maternal and fetal hypothalamic-pituitary-adrenal axes during pregnancy and postpartum. Annals of the New York Academy of Sciences. 2003;997(1):136-49.
135. Sandman CA, Glynn L, Schetter CD, Wadhwa P, Garite T, Chicz-DeMet A, et al. Elevated maternal cortisol early in pregnancy predicts third trimester levels of placental corticotropin releasing hormone (CRH): priming the placental clock. Peptides. 2006;27(6):1457-63.
136. Davis EP, Sandman CA. The timing of prenatal exposure to maternal cortisol and psychosocial stress is associated with human infant cognitive development. Child Development. 2010;81(1):131-48.
137. Ballard PL. Hormonal influences during fetal lung development. Ciba Foundation Symposium. 1980;78:251-74.
138. Charil A, Laplante DP, Vaillancourt C, King S. Prenatal stress and brain development. Brain Research Reviews. 2010;65(1):56-79.
139. Borzsonyi B, Demendi C, Pajor A, Rigo J, Jr., Marosi K, Agota A, et al. Gene expression patterns of the 11beta-hydroxysteroid dehydrogenase 2 enzyme in human placenta from intrauterine growth restriction: the role of impaired feto-maternal glucocorticoid metabolism. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2012;161(1):12-7.
140. Dy J, Guan H, Sampath-Kumar R, Richardson BS, Yang K. Placental 11beta-hydroxysteroid dehydrogenase type 2 is reduced in pregnancies complicated with idiopathic intrauterine growth Restriction: evidence that this is associated with an

attenuated ratio of cortisone to cortisol in the umbilical artery. Placenta. 2008;29(2):193-200.

141. Demendi C, Borzsonyi B, Pajor A, Rigo J, Jr., Nagy ZB, Szentpeteri I, et al. Abnormal fetomaternal glucocorticoid metabolism in the background of premature delivery: placental expression patterns of the 11beta-hydroxysteroid dehydrogenase 2 gene. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2012;165(2):210-4.
142. Mericq V, Medina P, Kakarieka E, Marquez L, Johnson MC, Iniguez G. Differences in expression and activity of 11beta-hydroxysteroid dehydrogenase type 1 and 2 in human placentas of term pregnancies according to birth weight and gender. European Journal of Endocrinology. 2009;161(3):419-25.
143. Green BB, Armstrong DA, Lesseur C, Paquette AG, Guerin DJ, Kwan LE, et al. The Role of Placental 11-Beta Hydroxysteroid Dehydrogenase Type 1 and Type 2 Methylation on Gene Expression and Infant Birth Weight. Biology of Reproduction. 2015;92(6):149.
144. Kajantie E, Dunkel L, Turpeinen U, Stenman UH, Andersson S. Placental 11beta-HSD2 activity, early postnatal clinical course, and adrenal function in extremely low birth weight infants. Pediatric Research. 2006;59(4 Pt 1):575-8.
145. Seckl JR, Holmes MC. Mechanisms of disease: glucocorticoids, their placental metabolism and fetal 'programming' of adult pathophysiology. Nature Reviews Endocrinology. 2007;3(6):479-88.
146. Laszlo KD, Liu XQ, Svensson T, Wikstrom AK, Li J, Olsen J, et al. Psychosocial stress related to the loss of a close relative the year before or during pregnancy and risk of preeclampsia. Hypertension. 2013;62(1):183-9.

147. Hogg K, Blair JD, McFadden DE, von Dadelszen P, Robinson WP. Early onset pre-eclampsia is associated with altered DNA methylation of cortisol-signalling and steroidogenic genes in the placenta. PLoS One. 2013;8(5):e62969.

148. Zhang D, Zeng J, Miao X, Liu H, Ge L, Huang W, et al. Glucocorticoid exposure induces preeclampsia via dampening 1, 25-dihydroxyvitamin D 3. Hypertension Research. 2018;41(2):104.

149. Zhang D, Liu H, Zeng J, Miao X, Huang W, Chen H, et al. Glucocorticoid exposure in early placentation induces preeclampsia in rats via interfering trophoblast development. General and Comparative Endocrinology. 2016;225:61-70.

150. Smith A, Witte E, McGee D, Knott J, Narang K, Racicot K. Cortisol inhibits CSF2 and CSF3 via DNA methylation and inhibits invasion in first-trimester trophoblast cells. American Journal of Reproductive Immunology. 2017;78(5).

151. Kisanga EP, Tang Z, Guller S, Whirledge S. Glucocorticoid signaling regulates cell invasion and migration in the human first-trimester trophoblast cell line Sw. 71. American Journal of Reproductive Immunology. 2018:e12974.

152. Giurgescu C. Are maternal cortisol levels related to preterm birth? Journal of Obstetric, Gynecologic, & Neonatal Nursing. 2009;38(4):377-90.

153. Elenkov IJ, Chrousos GP. Stress hormones, proinflammatory and antiinflammatory cytokines, and autoimmunity. Annals of the New York Academy of Sciences. 2002;966:290-303.

154. Elenkov IJ. Glucocorticoids and the Th1/Th2 balance. Annals of the New York Academy of Sciences. 2004;1024:138-46.

155. Coussons-Read ME, Okun ML, Schmitt MP, Giese S. Prenatal stress alters cytokine levels in a manner that may endanger human pregnancy. Psychosomatic Medicine. 2005;67(4):625-31.

156. Witek-Janusek L, Gabram S, Mathews HL. Psychologic stress, reduced NK cell activity, and cytokine dysregulation in women experiencing diagnostic breast biopsy. Psychoneuroendocrinology. 2007;32(1):22-35.

157. Azarmy M, Abolfathi, A, Ahmadijouybari, T, Ataee, M, Lotfi, B & Aghaei, A. Comparison of serum and urinary cortisol levels in women with gestational diabetes and healthy pregnant women. Iranian Journal of Obstetrics, Gynaecology, and Infertility. 2015;17(135):16-25.

158. Hornnes PJ, Kuhl C. Gastrointestinal hormones and cortisol in normal pregnant women and women with gestational diabetes. Acta Endocrinologica. Supplementum (Copenhagen). 1986;277:24-6.

159. Ehlert U, Patalla U, Kirschbaum C, Piedmont E, Hellhammer DH. Postpartum blues: salivary cortisol and psychological factors. Journal of Psychosomatic Research. 1990;34(3):319-25.

160. Handley SL, Dunn TL, Baker JM, Cockshott C, Gould S. Mood changes in puerperium, and plasma tryptophan and cortisol concentrations. BMJ. 1977;2(6078):18-20.

161. Okano T, Nomura J. Endocrine study of the maternity blues. Progress in Neuro-Psychopharmacology & Biological Psychiatry. 1992;16(6):921-32.

162. Taylor A, Littlewood J, Adams D, Dore C, Glover V. Serum cortisol levels are related to moods of elation and dysphoria in new mothers. Psychiatry Research. 1994;54(3):241-7.

163. Abou-Saleh MT, Ghubash R, Karim L, Krymski M, Bhai I. Hormonal aspects of postpartum depression. Psychoneuroendocrinology. 1998;23(5):465-75.

164. Brinsmead M, Smith R, Singh B, Lewin T, Owens P. Peripartum concentrations of beta endorphin and cortisol and maternal mood states. Australian and New Zealand Journal of Obstetrics and Gynaecology. 1985;25(3):194-7.

165. Davis EP, Glynn LM, Schetter CD, Hobel C, Chicz-Demet A, Sandman CA. Prenatal exposure to maternal depression and cortisol influences infant temperament. Journal of the American Academy of Child and Adolescent Psychiatry. 2007;46(6):737-46.

166. Field T, Diego MA, Hernandez-Reif M, Figueiredo B, Ascencio A, Schanberg S, et al. Prenatal dysthymia versus major depression effects on maternal cortisol and fetal growth. Depression and Anxiety. 2008;25(6):E11-6.

167. Figueiredo B, Costa R. Mother's stress, mood and emotional involvement with the infant: 3 months before and 3 months after childbirth. Archives of Women's Mental Health. 2009;12(3):143-53.

168. Harris B, Lovett L, Newcombe RG, Read GF, Walker R, Riad-Fahmy D. Maternity blues and major endocrine changes: Cardiff puerperal mood and hormone study II. BMJ. 1994;308(6934):949-53.

169. Kuevi V, Causon R, Dixson AF, Everard DM, Hall JM, Hole D, et al. Plasma amine and hormone changes in 'post-partum blues'. Clinical Endocrinology (Oxford). 1983;19(1):39-46.

170. O'Keane V, Lightman S, Patrick K, Marsh M, Papadopoulos AS, Pawlby S, et al. Changes in the maternal hypothalamic-pituitary-adrenal axis during the early puerperium may be related to the postpartum 'blues'. Journal of Neuroendocrinology. 2011;23(11):1149-55.

171. Smith R, Cubis J, Brinsmead M, Lewin T, Singh B, Owens P, et al. Mood changes, obstetric experience and alterations in plasma cortisol, beta-endorphin and corticotrophin releasing hormone during pregnancy and the puerperium. Journal of Psychosomatic Research. 1990;34(1):53-69.

172. Susman EJ, Schmeelk KH, Worrall BK, Granger DA, Ponirakis A, Chrousos GP. Corticotropin-releasing hormone and cortisol: longitudinal associations with depression and antisocial behavior in pregnant adolescents. Journal of the American Academy of Child and Adolescent Psychiatry. 1999;38(4):460-7.

173. Tsubouchi H, Nakai Y, Toda M, Morimoto K, Chang YS, Ushioda N, et al. Change of salivary stress marker concentrations during pregnancy: maternal depressive status suppress changes of those levels. Journal of Obstetrics and Gynaecology Research. 2011;37(8):1004-9.

174. Seth S, Lewis AJ, Galbally M. Perinatal maternal depression and cortisol function in pregnancy and the postpartum period: a systematic literature review. BMC Pregnancy Childbirth. 2016;16(1):124.

175. Romero R, Espinoza J, Gonçalves LF, Kusanovic JP, Friel LA, Nien JK, editors. Inflammation in preterm and term labour and delivery. Seminars in Fetal and Neonatal Medicine; 2006: Elsevier.

176. Challis JR, Lockwood CJ, Myatt L, Norman JE, Strauss III JF, Petraglia F. Inflammation and pregnancy. Reproductive Sciences. 2009;16(2):206-15.

177. Orsi N, Tribe R. Cytokine networks and the regulation of uterine function in pregnancy and parturition. Journal of Neuroendocrinology. 2008;20(4):462-9.

178. Szekeres-Bartho J, Wilczynski JR, Basta P, Kalinka J. Role of progesterone and progestin therapy in threatened abortion and preterm labour. Frontiers in Bioscience. 2008;13(5):1981-90.

179. Elovitz MA, Mrinalini C. Can medroxyprogesterone acetate alter Toll-like receptor expression in a mouse model of intrauterine inflammation? American Journal of Obstetrics and Gynecology. 2005;193(3):1149-55.

180. Oner C, Schatz F, Kizilay G, Murk W, Buchwalder LF, Kayisli UA, et al. Progestin-inflammatory cytokine interactions affect matrix metalloproteinase-1 and-3 expression in term decidual cells: implications for treatment of chorioamnionitis-induced preterm delivery. The Journal of Clinical Endocrinology & Metabolism. 2008;93(1):252-9.

181. Straub RH. The complex role of estrogens in inflammation. Endocrine Reviews. 2007;28(5):521-74.

182. Salem ML. Estrogen, a double-edged sword: modulation of TH1-and TH2-mediated inflammations by differential regulation of TH1/TH2 cytokine production. Current Drug Targets-Inflammation & Allergy. 2004;3(1):97-104.

183. Li MO, Flavell RA. TGF-beta: a master of all T cell trades. Cell. 2008;134(3):392-404.

184. Li MO, Wan YY, Sanjabi S, Robertson AK, Flavell RA. Transforming growth factor-beta regulation of immune responses. Annual Review of Immunology. 2006;24:99-146.

185. Derynck R, Jarrett JA, Chen EY, Eaton DH, Bell JR, Assoian RK, et al. Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells. Nature. 1985;316(6030):701-5.

186. Rifkin DB. Latent transforming growth factor-beta (TGF-beta) binding proteins: orchestrators of TGF-beta availability. Journal of Biological Chemistry. 2005;280(9):7409-12.

187. Annes JP, Munger JS, Rifkin DB. Making sense of latent TGFbeta activation. J Journal of Cell Science. 2003;116(Pt 2):217-24.

188. Massagué J. TGFβ signalling in context. Nature Reviews Molecular Cell Biology. 2012;13:616.

189. Derynck R, Jarrett JA, Chen EY, Eaton DH, Bell JR, Assoian RK, et al. Human transforming growth factor-β complementary DNA sequence and expression in normal and transformed cells. Nature. 1985;316(6030):701.

190. Xu X, Zheng L, Yuan Q, Zhen G, Crane JL, Zhou X, et al. Transforming growth factor-β in stem cells and tissue homeostasis. Bone Research. 2018;6(1):2.

191. Jian H, Shen X, Liu I, Semenov M, He X, Wang X-F. Smad3-dependent nuclear translocation of β-catenin is required for TGF-β1-induced proliferation of bone marrow-derived adult human mesenchymal stem cells. Genes & Development. 2006;20(6):666-74.

192. Boland GM, Perkins G, Hall DJ, Tuan RS. Wnt 3a promotes proliferation and suppresses osteogenic differentiation of adult human mesenchymal stem cells. Journal of Cellular Biochemistry. 2004;93(6):1210-30.

193. Baksh D, Boland GM, Tuan RS. Cross-talk between Wnt signaling pathways in human mesenchymal stem cells leads to functional antagonism during osteogenic differentiation. Journal of Cellular Biochemistry. 2007;101(5):1109-24.

194. Meng X-m, Nikolic-Paterson DJ, Lan HY. TGF-β: the master regulator of fibrosis. Nature Reviews Nephrology. 2016;12(6):325.

195. Ducray JF, Naicker T, Moodley J. Pilot study of comparative placental morphometry in pre-eclamptic and normotensive pregnancies suggests possible maladaptations of the fetal component of the placenta. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2011;156(1):29-34.

196. Devisme L, Merlot B, Ego A, Houfflin-Debarge V, Deruelle P, Subtil D. A case–control study of placental lesions associated with pre-eclampsia. International Journal of Gynecology & Obstetrics. 2013;120(2):165-8.

197. Border WA, Okuda S, Languino LR, Sporn MB, Ruoslahti E. Suppression of experimental glomerulonephritis by antiserum against transforming growth factor β1. Nature. 1990;346(6282):371.

198. Leask A. Potential therapeutic targets for cardiac fibrosis: TGFβ, angiotensin, endothelin, CCN2, and PDGF, partners in fibroblast activation. Circulation Research. 2010;106(11):1675-80.

199. Kendall RT, Feghali-Bostwick CA. Fibroblasts in fibrosis: novel roles and mediators. Frontiers in Pharmacology. 2014;5:123.

200. Li Q. Transforming growth factor beta signaling in uterine development and function. Journal of Animal Science and Biotechnology. 2014;5(1):52.

201. Jones RL, Stoikos C, Findlay JK, Salamonsen LA. TGF-beta superfamily expression and actions in the endometrium and placenta. Reproduction. 2006;132(2):217-32.

202. Fafet P, Rebouissou C, Maudelonde T, Vignais ML. Opposite effects of transforming growth factor-beta activation and rho-associated kinase inhibition on human trophoblast migration in a reconstituted placental-endometrial coculture system. Endocrinology. 2008;149(9):4475-85.

203. Graham CH, Lala PK. Mechanism of control of trophoblast invasion in situ. Journal of Cellular Physiology. 1991;148(2):228-34.

204. Graham CH, Lysiak JJ, McCrae KR, Lala PK. Localization of transforming growth factor-beta at the human fetal-maternal interface: role in trophoblast growth and differentiation. Biology of Reproduction. 1992;46(4):561-72.

205. Lash GE, Otun HA, Innes BA, Bulmer JN, Searle RF, Robson SC. Inhibition of trophoblast cell invasion by TGFB1, 2, and 3 is associated with a decrease in active proteases. Biology of Reproduction. 2005;73(2):374-81.

206. Grady WM. Transforming growth factor-beta, Smads, and cancer. Clinical Cancer Research. 2005;11(9):3151-4.

207. Li Y, Xu Q, Zhang Z, Liu S, Shi C, Tan Y. The impact of TGF-beta1 on the mRNA expression of TbetaR I, TbetaR II, Smad4 and the invasiveness of the JEG-3 placental choriocarcinoma cell line. Oncology Letters. 2012;4(6):1344-8.

208. Huang Z, Li S, Fan W, Ma Q. Transforming growth factor beta1 promotes invasion of human JEG-3 trophoblast cells via TGF-beta/Smad3 signaling pathway. Oncotarget. 2017;8(20):33560-70.

209. Gomez R, Ghezzi F, Romero R, Munoz H, Tolosa JE, Rojas I. Premature labor and intra-amniotic infection. Clinical aspects and role of the cytokines in diagnosis and pathophysiology. Clinics in Perinatology. 1995;22(2):281-342.

210. Li Y, Xiao B, Qiu W, Yang L, Hu B, Tian X, et al. Altered expression of CD4(+)CD25(+) regulatory T cells and its 5-HT(1a) receptor in patients with major depression disorder. Journal of Affective Disorders. 2010;124(1-2):68-75.

211. Musil R, Schwarz MJ, Riedel M, Dehning S, Cerovecki A, Spellmann I, et al. Elevated macrophage migration inhibitory factor and decreased transforming growth factor-beta levels in major depression--no influence of celecoxib treatment. Journal of Affective Disorders. 2011;134(1-3):217-25.

212. Myint AM, Leonard BE, Steinbusch HW, Kim YK. Th1, Th2, and Th3 cytokine alterations in major depression. Journal of Affective Disorders. 2005;88(2):167-73.

213. Sutcigil L, Oktenli C, Musabak U, Bozkurt A, Cansever A, Uzun O, et al. Pro- and anti-inflammatory cytokine balance in major depression: effect of sertraline therapy. Clinical and Developmental Immunology. 2007;2007:76396.

214. Esmatjes E, Flores L, Lario S, Claria J, Cases A, Inigo P, et al. Smoking increases serum levels of transforming growth factor-beta in diabetic patients. Diabetes Care. 1999;22(11):1915-6.

215. Pfeiffer A, Middelberg-Bisping K, Drewes C, Schatz H. Elevated plasma levels of transforming growth factor-beta 1 in NIDDM. Diabetes Care. 1996;19(10):1113-7.

216. Shankland SJ, Scholey JW, Ly H, Thai K. Expression of transforming growth factor-beta 1 during diabetic renal hypertrophy. Kidney International. 1994;46(2):430-42.

217. Yener S, Demir T, Akinci B, Bayraktar F, Kebapcilar L, Ozcan MA, et al. Transforming growth factor-beta 1 levels in women with prior history of gestational diabetes mellitus. Diabetes Research and Clinical Practice. 2007;76(2):193-8.

218. St-Pierre J, Laurent L, King S, Vaillancourt C. Effects of prenatal maternal stress on serotonin and fetal development. Placenta. 2016;48 Suppl 1:S66-S71.

219. Berger M, Gray JA, Roth BL. The expanded biology of serotonin. Annual review of Medicine. 2009;60:355-66.

220. Rapport MM, Green AA, Page IH. Serum vasoconstrictor (serotonin) IV. Isolation and characterization. Journal of Biological Chemistry. 1948;176(3):1243-51.

221. Coleman JA, Green EM, Gouaux E. X-ray structures and mechanism of the human serotonin transporter. Nature. 2016;532(7599):334.

222. Dürk T, Duerschmied D, Müller T, Grimm M, Reuter S, Vieira RP, et al. Production of serotonin by tryptophan hydroxylase 1 and release via platelets contribute to allergic airway inflammation. American Journal of Respiratory and Critical Care Medicine. 2013;187(5):476-85.
223. Racke K, Reimann A, Schwörer H, Kilbinger H. Regulation of 5-HT release from enterochromaffin cells. Behavioural Brain Research. 1995;73(1-2):83-7.
224. Mercado CP, Kilic F. Molecular mechanisms of SERT in platelets: regulation of plasma serotonin levels. Molecular Interventions. 2010;10(4):231.
225. Cooper A, Woulfe D, Kilic F. Post-translational modifications of serotonin transporter. Pharmacological Research. 2018.
226. Kristensen AS, Andersen J, Jørgensen TN, Sørensen L, Eriksen J, Loland CJ, et al. SLC6 neurotransmitter transporters: structure, function, and regulation. Pharmacological Reviews. 2011;63(3):585-640.
227. Bröer S, Gether U. The solute carrier 6 family of transporters. British Journal of Pharmacology. 2012;167(2):256-78.
228. Ozaslan D, Wang S, Ahmed BA, Kocabas AM, McCastlain JC, Bene A, et al. Glycosyl modification facilitates homo-and hetero-oligomerization of the serotonin transporter: a specific role for sialic acid residues. Journal of Biological Chemistry. 2003.
229. Zhang Y-W, Turk BE, Rudnick G. Control of serotonin transporter phosphorylation by conformational state. Proceedings of the National Academy of Sciences. 2016;113(20):E2776-E83.
230. Chen J-G, Liu-Chen S, Rudnick G. External cysteine residues in the serotonin transporter. Biochemistry. 1997;36(6):1479-86.

231. Kilic F, Rudnick G. Oligomerization of serotonin transporter and its functional consequences. Proceedings of the National Academy of Sciences. 2000;97(7):3106-11.

232. Auda GR, Kirk SH, Billett MA, Billett EE. Localization of monoamine oxidase mRNA in human placenta. Journal of Histochemistry & Cytochemistry. 1998;46(12):1393-400.

233. Huang WQ, Zhang CL, Di XY, Zhang RQ. Studies on the localization of 5-hydroxytryptamine and its receptors in human placenta. Placenta. 1998;19(8):655-61.

234. Bottalico B, Larsson I, Brodszki J, Hernandez-Andrade E, Casslen B, Marsal K, et al. Norepinephrine transporter (NET), serotonin transporter (SERT), vesicular monoamine transporter (VMAT2) and organic cation transporters (OCT1, 2 and EMT) in human placenta from pre-eclamptic and normotensive pregnancies. Placenta. 2004;25(6):518-29.

235. Laurent L, Deroy K, St-Pierre J, Cote F, Sanderson JT, Vaillancourt C. Human placenta expresses both peripheral and neuronal isoform of tryptophan hydroxylase. Biochimie. 2017;140:159-65.

236. Deroy K, St-Pierre, J, Thomas Sanderson, J, Vaillancourt, C. Human villous and extravillous trophoblast cells produce serotonin de novo. Placenta. 2011;32:1.

237. Kliman HJ, Quaratella SB, Setaro AC, Siegman EC, Subha ZT, Tal R, et al. Pathway of Maternal Serotonin to the Human Embryo and Fetus. Endocrinology. 2018;159(4):1609-29.

238. Cote F, Fligny C, Bayard E, Launay JM, Gershon MD, Mallet J, et al. Maternal serotonin is crucial for murine embryonic development. Proceedings of the National Academy of Sciences USA. 2007;104(1):329-34.

239. Oufkir T, Arseneault M, Sanderson JT, Vaillancourt C. The 5-HT 2A serotonin receptor enhances cell viability, affects cell cycle progression and activates MEK-ERK1/2 and JAK2-STAT3 signalling pathways in human choriocarcinoma cell lines. Placenta. 2010;31(5):439-47.

240. Hadden C, Fahmi T, Cooper A, Savenka AV, Lupashin VV, Roberts DJ, et al. Serotonin transporter protects the placental cells against apoptosis in caspase 3-independent pathway. Journal of Cellular Physiology. 2017;232(12):3520-9.

241. Kim H, Toyofuku Y, Lynn FC, Chak E, Uchida T, Mizukami H, et al. Serotonin regulates pancreatic beta cell mass during pregnancy. Nature Medicine. 2010;16(7):804-8.

242. Kim MS, Lee WS, Jeong J, Kim SJ, Jin W. Induction of metastatic potential by TrkB via activation of IL6/JAK2/STAT3 and PI3K/AKT signaling in breast cancer. Oncotarget. 2015;6(37):40158-71.

243. Mercado CP, Byrum S, Beggs ML, Ziu E, Singh P, Raj VR, et al. Impact of elevated plasma serotonin on global gene expression of murine megakaryocytes. PLoS One. 2013;8(8):e72580.

244. Nebigil CG, Etienne N, Messaddeq N, Maroteaux L. Serotonin is a novel survival factor of cardiomyocytes: mitochondria as a target of 5-HT2B receptor signaling. The FASEB Journal. 2003;17(10):1373-5.

245. Balsells M, Garcia-Patterson A, Gich I, Corcoy R. Major congenital malformations in women with gestational diabetes mellitus: a systematic review and meta-analysis. Diabetes/Metabolism Research and Reviews. 2012;28(3):252-7.

246. Chambers CD, Hernandez-Diaz S, Van Marter LJ, Werler MM, Louik C, Jones KL, et al. Selective serotonin-reuptake inhibitors and risk of persistent pulmonary

hypertension of the newborn. New England Journal of Medicine. 2006;354(6):579-87.

247. Huybrechts KF, Bateman BT, Palmsten K, Desai RJ, Patorno E, Gopalakrishnan C, et al. Antidepressant use late in pregnancy and risk of persistent pulmonary hypertension of the newborn. JAMA. 2015;313(21):2142-51.

248. Huybrechts KF, Hernandez-Diaz S, Avorn J. Antidepressant use in pregnancy and the risk of cardiac defects. New England Journal of Medicine. 2014;371(12):1168-9.

249. Wemakor A, Casson K, Garne E, Bakker M, Addor MC, Arriola L, et al. Selective serotonin reuptake inhibitor antidepressant use in first trimester pregnancy and risk of specific congenital anomalies: a European register-based study. European Journal of Epidemiology. 2015;30(11):1187-98.

250. Armant DR A-PM, Kuhn DM. Serotonergic Signaling by Preimplantation Embryos Impacts Subsequent Trophoblast Differentiation and Pregnancy Failure. Biology of Reproduction. 2012;87(1):1.

251. Poulson E, Botros M, Robson JM. Effect of 5-hydroxytryptamine and iproniazid on pregnancy. Science. 1960;131(3407):1101-2.

252. Bolte AC, van Geijn HP, Dekker GA. Pathophysiology of preeclampsia and the role of serotonin. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2001;95(1):12-21.

253. Senior JB, Fahim I, Sullivan FM, Robson JM. Possible Role of 5-Hydroxytryptamine in Toxaemia of Pregnancy. Lancet. 1963;2(7307):553-4.

254. Filshie GM, Maynard P, Hutter C, Cooper JC, Robinson G, Rubin P. Urinary 5-hydroxyindole acetate concentration in pregnancy induced hypertension. BMJ. 1992;304(6836):1223.

255. Ishii Y, Kanai H, Maezawa A, Tsuchida A, Yano S, Naruse T. Evaluation of intraplatelet and urinary 5-hydroxytryptamine (5-HT), and urinary 5-hydroxyindoleacetic acid (5-HIAA) levels in patients with toxemia of pregnancy. Research Communications in Chemical Pathology and Pharmacology. 1993;80(1):21-40.

256. Hajduch E, Rencurel F, Balendran A, Batty IH, Downes CP, Hundal HS. Serotonin (5-Hydroxytryptamine), a novel regulator of glucose transport in rat skeletal muscle. Journal of Biological Chemistry. 1999;274(19):13563-8.

257. Lam DD, Heisler LK. Serotonin and energy balance: molecular mechanisms and implications for type 2 diabetes. Expert Rev Mol Med. 2007;9(5):1-24.

258. Rattigan S, Clark MG, Barrett EJ. Acute vasoconstriction-induced insulin resistance in rat muscle in vivo. Diabetes. 1999;48(3):564-9.

259. Rattigan S, Dora KA, Colquhoun EQ, Clark MG. Serotonin-mediated acute insulin resistance in the perfused rat hindlimb but not in incubated muscle: a role for the vascular system. Life Sciences. 1993;53(20):1545-55.

260. Isaac R, Boura-Halfon S, Gurevitch D, Shainskaya A, Levkovitz Y, Zick Y. Selective serotonin reuptake inhibitors (SSRIs) inhibit insulin secretion and action in pancreatic beta cells. Journal of Biomedical Chemistry. 2013;288(8):5682-93.

261. Li Y, Hadden C, Singh P, Mercado CP, Murphy P, Dajani NK, et al. GDM-associated insulin deficiency hinders the dissociation of SERT from ERp44 and down-regulates placental 5-HT uptake. Proceedings of the National Academy of Sciences. 2014;111(52):E5697-E705.

262. Kozhimannil KB, Pereira MA, Harlow BL. Association between diabetes and perinatal depression among low-income mothers. JAMA. 2009;301(8):842-7.

263. Mezuk B, Eaton WW, Albrecht S, Golden SH. Depression and type 2 diabetes over the lifespan: a meta-analysis. Diabetes Care. 2008;31(12):2383-90.

264. Gold PW. The organization of the stress system and its dysregulation in depressive illness. Molecular Psychiatry. 2015;20(1):32-47.

265. Roy A, Campbell MK. A unifying framework for depression: bridging the major biological and psychosocial theories through stress. Clinical and Investigative Medicine. 2013;36(4):E170-90.

266. Verduijn J, Milaneschi Y, Schoevers RA, van Hemert AM, Beekman AT, Penninx BW. Pathophysiology of major depressive disorder: mechanisms involved in etiology are not associated with clinical progression. Translational Psychiatry. 2015;5:e649.

267. Yonkers KA, Vigod S, Ross LE. Diagnosis, pathophysiology, and management of mood disorders in pregnant and postpartum women. Obstetrics & Gynecology. 2011;117(4):961-77.

268. Rose'meyer R. A review of the serotonin transporter and prenatal cortisol in the development of autism spectrum disorders. Molecular Autism. 2013;4(1):37.

269. Couch Y, Anthony DC, Dolgov O, Revischin A, Festoff B, Santos AI, et al. Microglial activation, increased TNF and SERT expression in the prefrontal cortex define stress-altered behaviour in mice susceptible to anhedonia. Brain, Behavior, and Immunity. 2013;29:136-46.

270. Tafet GE, Toister-Achituv M, Shinitzky M. Enhancement of serotonin uptake by cortisol: a possible link between stress and depression. Cognitive, Affective, & Behavioral Neuroscience. 2001;1(1):96-104.

271. Zhang J, Fan Y, Li Y, Zhu H, Wang L, Zhu MY. Chronic social defeat up-regulates expression of the serotonin transporter in rat dorsal raphe nucleus and

projection regions in a glucocorticoid-dependent manner. Journal of Neurochemistry. 2012;123(6):1054-68.

272. Glatz K, Mossner R, Heils A, Lesch KP. Glucocorticoid-regulated human serotonin transporter (5-HTT) expression is modulated by the 5-HTT gene-promotor-linked polymorphic region. Journal of Neurochemistry. 2003;86(5):1072-8.

273. Lau T, Heimann F, Bartsch D, Schloss P, Weber T. Nongenomic, glucocorticoid receptor-mediated regulation of serotonin transporter cell surface expression in embryonic stem cell derived serotonergic neurons. Neurosciene Letters. 2013;554:115-20.

274. Ambros V. The functions of animal microRNAs. Nature. 2004;431(7006):350-5.

275. Baudry A, Mouillet-Richard S, Schneider B, Launay JM, Kellermann O. miR-16 targets the serotonin transporter: a new facet for adaptive responses to antidepressants. Science. 2010;329(5998):1537-41.

276. Maccani MA, Avissar-Whiting M, Banister CE, McGonnigal B, Padbury JF, Marsit CJ. Maternal cigarette smoking during pregnancy is associated with downregulation of miR-16, miR-21, and miR-146a in the placenta. Epigenetics. 2010;5(7):583-9.

277. Moya PR, Wendland JR, Salemme J, Fried RL, Murphy DL. miR-15a and miR-16 regulate serotonin transporter expression in human placental and rat brain raphe cells. The International Journal of Neuropsychopharmacology. 2013;16(3):621-9.

278. Cui SF, Li W, Niu J, Zhang CY, Chen X, Ma JZ. Acute responses of circulating microRNAs to low-volume sprint interval cycling. Front Physiol. 2015;6:311.

279. Shang H, Sun L, Braun T, Si Q, Tong J. Revealing the action mechanisms of dexamethasone on the birth weight of infant using RNA-sequencing data of trophoblast cells. Medicine (Baltimore). 2018;97(4):e9653.

280. Kajikawa K, Yasui W, Sumiyoshi H, Yoshida K, Nakayama H, Ayhan A, et al. Expression of epidermal growth factor in human tissues. Immunohistochemical and biochemical analysis. Virchows Archiv A Pathological Anatomy and Histology. 1991;418(1):27-32.

281. Armant DR, Fritz R, Kilburn BA, Kim YM, Nien JK, Maihle NJ, et al. Reduced expression of the epidermal growth factor signaling system in preeclampsia. Placenta. 2015;36(3):270-8.

282. Cui XF, Zhou WM, Yang Y, Zhou J, Li XL, Lin L, et al. Epidermal growth factor upregulates serotonin transporter and its association with visceral hypersensitivity in irritable bowel syndrome. World Journal of Gastroenterology. 2014;20(37):13521-9.

283. Taketani Y, Mizuno M. Evidence for direct regulation of epidermal growth factor receptors by steroid hormones in human endometrial cells. Human Reproduction. 1991;6(10):1365-9.

284. Conrad KP, Benyo DF, Westerhausen-Larsen A, Miles TM. Expression of erythropoietin by the human placenta. The FASEB Journal. 1996;10(7):760-8.

285. Fairchild Benyo D, Conrad KP. Expression of the erythropoietin receptor by trophoblast cellsin the human placenta. Biology of Reproduction. 1999;60(4):861-70.

286. Ji YQ, Zhang YQ, Li MQ, Du MR, Wei WW, Li DJ. EPO improves the proliferation and inhibits apoptosis of trophoblast and decidual stromal cells

through activating STAT-5 and inactivating p38 signal in human early pregnancy. International Journal of Clinical and Experimental Pathology. 2011;4(8):765-74.

287. Dengler VL, Galbraith M, Espinosa JM. Transcriptional regulation by hypoxia inducible factors. Critical Reviews in Biochemistry and Molecular Biology. 2014;49(1):1-15.

288. Hiden U, Eyth CP, Majali-Martinez A, Desoye G, Tam-Amersdorfer C, Huppertz B, et al. Expression of matrix metalloproteinase 12 is highly specific for non-proliferating invasive trophoblasts in the first trimester and temporally regulated by oxygen-dependent mechanisms including HIF-1A. Histochemistry and Cell Biology. 2018;149(1):31-42.

289. Dudek N, Vantrease, JE, & Scrogin, KE. Serotonin 5-HT1A receptor activation inhibits HIF-1α expression in a rat embryonic serotonergic cell line. The FASEB Journal. 2011;25(1).

290. Issler O, Haramati S, Paul ED, Maeno H, Navon I, Zwang R, et al. MicroRNA 135 is essential for chronic stress resiliency, antidepressant efficacy, and intact serotonergic activity. Neuron. 2014;83(2):344-60.

291. Chim SS, Shing TK, Hung EC, Leung TY, Lau TK, Chiu RW, et al. Detection and characterization of placental microRNAs in maternal plasma. Clinical Chemistry. 2008;54(3):482-90.

292. Maccani MA, Padbury JF, Marsit CJ. miR-16 and miR-21 expression in the placenta is associated with fetal growth. PLoS One. 2011;6(6):e21210.

293. Tamaru S, Mizuno Y, Tochigi H, Kajihara T, Okazaki Y, Okagaki R, et al. MicroRNA-135b suppresses extravillous trophoblast-derived HTR-8/SVneo cell invasion by directly down regulating CXCL12 under low oxygen conditions. Biochemical and Biophysical Research Communications. 2015;461(2):421-6.

294. Zhang L, Sun ZJ, Bian Y, Kulkarni AB. MicroRNA-135b acts as a tumor promoter by targeting the hypoxia-inducible factor pathway in genetically defined mouse model of head and neck squamous cell carcinoma. Cancer Letters. 2013;331(2):230-8.

295. Nazir S, Kumar A, Chatterjee I, Anbazhagan AN, Gujral T, Priyamvada S, et al. Mechanisms of intestinal serotonin transporter (SERT) upregulation by TGF-β1 induced non-Smad pathways. PloS One. 2015;10(5):e0120447.

296. Gill RK KA, Anbazhagan AN, Singla A, Nazir SF, Priyamvada S, Alrefai WA & Saksena S. TGF-β1 upregulates serotonin transporter function and expression via PI3K and SMAD 3-mediated pathways. Gastroenterology. 2011;140(5):133.

297. Llobet-Navas D, Rodriguez-Barrueco R, Castro V, Ugalde AP, Sumazin P, Jacob-Sendler D, et al. The miR-424(322)/503 cluster orchestrates remodeling of the epithelium in the involuting mammary gland. Genes & Development. 2014;28(7):765-82.

298. Abdel-Salam OME, Baiuomy AR, Arbid MS. Studies on the anti-inflammatory effect of fluoxetine in the rat. Pharmacological Research. 2004;49(2):119-31.

299. Bianchi M, Sacerdote P, Panerai AE. Fluoxetine reduces inflammatory edema in the rat: Involvement of the pituitary-adrenal axis. European Journal of Pharmacology. 1994;263(1):81-4.

300. Bianchi M, Rossoni G, Sacerdote P, Panerai AE, Berti F. Effects of chlomipramine and fluoxetine on subcutaneous carrageenin-induced inflammation in the rat. Inflammation Research. 1995;44(11):466-9.

301. Sawynok J, Esser MJ, Reid AR. Peripheral antinociceptive actions of desipramine and fluoxetine in an inflammatory and neuropathic pain test in the rat. Pain. 1999;82(2):149-58.

302. Periyasamy S, Sánchez ER. Antagonism of glucocorticoid receptor transactivity and cell growth inhibition by transforming growth factor-β through AP-1-mediated transcriptional repression. The International Journal of Biochemistry & Cell Biology. 2002;34(12):1571-85.

303. Ichijo T, Voutetakis A, Cotrim AP, Bhattachryya N, Fujii M, Chrousos GP, et al. The Smad6-histone deacetylase 3 complex silences the transcriptional activity of the glucocorticoid receptor Potential clinical implications. Journal of Biological Chemistry. 2005;280(51):42067-77.

304. Salem S, Harris T, Mok J, Li MYS, Keenan C, Schuliga M, et al. Transforming growth factor-β impairs glucocorticoid activity in the A549 lung adenocarcinoma cell line. British Journal of Pharmacology. 2012;166(7):2036-48.

305. Peltier J, Perez J, Bellocq A, Escoubet B, Fouqueray B, Baud L. Transforming growth factor-β1 increases glucocorticoid binding and signaling in macrophages through a Smad-and activated protein-1–mediated process 1. Kidney International. 2003;63(6):2028-36.

306. Aversa S, Ondolo C, Abbadessa G, Piccione F, Carriero V, Fulcheri A, et al. Steroid resistance in nasal polyposis: role of glucocorticoid receptor and TGF-beta1. Rhinology. 2012;50(4):427-35.

307. Straszewski-Chavez SL, Abrahams VM, Alvero AB, Aldo PB, Ma Y, Guller S, et al. The isolation and characterization of a novel telomerase immortalized first trimester trophoblast cell line, Swan 71. Placenta. 2009;30(11):939-48.

308. Sullivan M. Endocrine cell lines from the placenta. Molecular and Cellular Endocrinology. 2004;228(1-2):103-19.

309. Kohler PO, Bridson WE. Isolation of hormone-producing clonal lines of human choriocarcinoma. The Journal of Clinical Endocrinology & Metabolism. 1971;32(5):683-7.

310. Mateos RM, Jimenez G, Alvarez-Gil C, Visiedo F, Rivera-Rodriguez F, et al. Excess Hydrocortisone Hampers Placental Nutrient Uptake Disrupting Cellular Metabolism. BioMed Research International. 2018;2018:11.

311. Žanka B-T, Nikola K, Miloš P, Ljiljana V. Choriocarcinoma cell line Response to Dexamethasone. Journal of Medical Biochemistry. 2010;29(2):107-13.

312. Tanaka H, Hirano F, Nomura Y, Miura T, Makino Y, Fukawa E, et al. Relative glucocorticoid potency revisited. Rheumatology International. 1994;14(1):9-12.

313. Tan Y, Xu Q, Li Y, Mao X, Zhang K. Crosstalk between the p38 and TGF-β signaling pathways through TβRI, TβRII and Smad3 expression in plancental choriocarcinoma JEG-3 cells. Oncology Letters. 2014;8(3):1307-11.

314. Khera A, Vanderlelie JJ, Holland O, Perkins AV. Overexpression of endogenous anti-oxidants with selenium supplementation protects trophoblast cells from reactive oxygen species-induced apoptosis in a Bcl-2-dependent manner. Biological Trace Element Research. 2017;177(2):394-403.

315. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods. 1983;65(1-2):55-63.

316. Koh JY, Choi DW. Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. Journal of Neuroscience Methods. 1987;20(1):83-90.

317. Cervellati F, Pavan B, Lunghi L, Manni E, Fabbri E, Mascoli C, et al. Betamethasone, progesterone and RU-486 (mifepristone) exert similar effects on

connexin expression in trophoblast-derived HTR-8/SVneo cells. Reproduction, Fertility and Development. 2011;23(2):319-28.

318. Li A, Felix JC, Minoo P, Amezcua CA, Jain JK. Effect of mifepristone on proliferation and apoptosis of Ishikawa endometrial adenocarcinoma cells. Fertility and Sterility. 2005;84(1):202-11.

319. Goyeneche A, Telleria C. Mifepristone inhibits ovarian cancer cell growth. Cancer Research. 2007;67(9 Supplement):4344.

320. Zhang S, Jonklaas J, Danielsen M. The glucocorticoid agonist activities of mifepristone (RU486) and progesterone are dependent on glucocorticoid receptor levels but not on EC50 values. Steroids. 2007;72(6-7):600-8.

321. Bo L, Xiangjun B, Haiping W. Effect of dexamethasone on expression of glucocorticoid receptor in human monocyte cell line THP-1. Journal of Huazhong University of Science and Technology [Medical Sciences]. 2006;26(1):25-7.

322. Pujols L, Mullol J, Pérez M, Roca-Ferrer J, Juan M, Xaubet A, et al. Expression of the human glucocorticoid receptor α and β isoforms in human respiratory epithelial cells and their regulation by dexamethasone. American Journal of Respiratory Cell and Molecular Biology. 2001;24(1):49-57.

323. Vogel C, Marcotte EM. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nature Reviews Genetics. 2012;13(4):227.

324. Guo Y, Xiao P, Lei S, Deng F, Xiao GG, Liu Y, et al. How is mRNA expression predictive for protein expression? A correlation study on human circulating monocytes. Acta Biochimica et Biophysica Sinica. 2008;40(5):426-36.

325. Greenbaum D, Colangelo C, Williams K, Gerstein M. Comparing protein abundance and mRNA expression levels on a genomic scale. Genome Biology. 2003;4(9):117.

326. Oskari H, Kimmo K, Horacio C, Irving S, Tapani L, Pekka L. Plasma concentrations and receptor binding of RU 486 and its metabolites in humans. The Journal of Steroid Biochemistry and Molecular Biology. 1987;26(2):279-84.

327. Gagne D, Pons M, Crastes A, Paulet D. Analysis of the relation between receptor binding affinity and antagonist efficacy of antiglucocorticoids. Journal of Steroid Biochemistry. 1986;25(3):315-22.

328. Geiss GK, Bumgarner RE, Birditt B, Dahl T, Dowidar N, Dunaway DL, et al. Direct multiplexed measurement of gene expression with color-coded probe pairs. Nature Biotechnology. 2008;26(3):317.

329. Chang KT, Goytain A, Tucker T, Karsan A, Lee C-H, Nielsen TO, et al. Development and evaluation of a Pan-Sarcoma fusion gene detection assay using the NanoString nCounter platform. The Journal of Molecular Diagnostics. 2018;20(1):63-77.

330. Dmitriev AD, Factor MI, Segal OL, Pavlova EV, Massino YS, Smirnova MB, et al. Western blot analysis of human and rat serotonin transporter in platelets and brain using site-specific antibodies: evidence that transporter undergoes endoproteolytic cleavage. Clinica Chimica Acta. 2005;356(1-2):76-94.

331. Mclane MW, Mccann U, Ricaurte G. Identifying the serotonin transporter signal in Western blot studies of the neurotoxic potential of MDMA and related drugs. Synapse. 2011;65(12):1368-72.

332. Ramamoorthy S, Cidlowski JA. Ligand-induced repression of glucocorticoid receptor gene is mediated by a NCoR1 repression complex formed by long range chromatin interactions with intragenic GREs. Molecular and Cellular Biology. 2013:MCB. 01151-12.

333. Patki M, Gadgeel S, Huang Y, McFall T, Shields AF, Matherly LH, et al. Glucocorticoid Receptor Status Is a Principal Determinant of Variability in the Sensitivity of Non–Small-Cell Lung Cancer Cells to Pemetrexed. Journal of Thoracic Oncology. 2014;9(4):519-26.

334. Cuffe JS, Saif Z, Perkins AV, Moritz KM, Clifton VL. Dexamethasone and sex regulate placental glucocorticoid receptor isoforms in mice. Journal of Endocrinology. 2017:JOE-17-0171.

335. Saif Z, Dyson RM, Palliser HK, Wright IM, Lu N, Clifton VL. Identification of eight different isoforms of the glucocorticoid receptor in guinea pig placenta: relationship to preterm delivery, sex and betamethasone exposure. PLoS One. 2016;11(2):e0148226.

336. Wu I, Shin S, Cao Y, Bender I, Jafari N, Feng G, et al. Selective glucocorticoid receptor translational isoforms reveal glucocorticoid-induced apoptotic transcriptomes. Cell Death & Disease. 2014;4(1):e453.

337. Bamberger CM, Bamberger A-M, de Castro M, Chrousos GP. Glucocorticoid receptor beta, a potential endogenous inhibitor of glucocorticoid action in humans. The Journal of Clinical Investigation. 1995;95(6):2435-41.

338. Schoonheim PJ, Chatzopoulou A, Schaaf MJ. The zebrafish as an in vivo model system for glucocorticoid resistance. Steroids. 2010;75(12):918-25.

339. Haarman E, Kaspers G, Pieters R, Rottier M, Veerman A. Glucocorticoid receptor alpha, beta and gamma expression vs in vitro glucocorticod resistance in childhood leukemia. Leukemia. 2004;18(3):530.

340. Beger C, Gerdes K, Lauten M, Tissing WJ, Fernandez-Munoz I, Schrappe M, et al. Expression and structural analysis of glucocorticoid receptor isoform gamma

in human leukaemia cells using an isoform-specific real-time polymerase chain reaction approach. British Journal of Haematology. 2003;122(2):245-52.

341. de Lange P, Segeren CM, Koper JW, Wiemer E, Sonneveld P, Brinkmann AO, et al. Expression in hematological malignancies of a glucocorticoid receptor splice variant that augments glucocorticoid receptor-mediated effects in transfected cells. Cancer Research. 2010;61(10):3937-41.
342. Wallace AD, Cidlowski JA. Proteasome-mediated glucocorticoid receptor degradation restricts transcriptional signaling by glucocorticoids. Journal of Biological Chemistry. 2001;276(46):42714-21.
343. Schaaf MJ, Cidlowski JA. Molecular mechanisms of glucocorticoid action and resistance. The Journal of Steroid Biochemistry and Molecular Biology. 2002;83(1-5):37-48.
344. McIntyre W, Samuels H. Triamcinolone acetonide regulates glucocorticoid-receptor levels by decreasing the half-life of the activated nuclear-receptor form. Journal of Biological Chemistry. 1985;260(1):418-27.
345. Vedeckis WV, Ali M, Allen HR. Regulation of glucocorticoid receptor protein and mRNA levels. Cancer Research. 1989;49(8 Supplement):2295s-302s.
346. Govindan M, Pothier F, Leclerc S, Palaniswami R, Xie B. Human glucocorticoid receptor gene promotor—homologous down regulation. The Journal of Steroid Biochemistry and Molecular Biology. 1991;40(1-3):317-23.
347. Chan CC, Lao TT, Ho PC, Sung EO, Cheung AN. The effect of mifepristone on the expression of steroid hormone receptors in human decidua and placenta: a randomized placebo-controlled double-blind study. The Journal of Clinical Endocrinology & Metabolism. 2003;88(12):5846-50.

348. Prasad HC, Zhu C-B, McCauley JL, Samuvel DJ, Ramamoorthy S, Shelton RC, et al. Human serotonin transporter variants display altered sensitivity to protein kinase G and p38 mitogen-activated protein kinase. Proceedings of the National Academy of Sciences. 2005;102(32):11545-50.

349. Kish SJ, Furukawa Y, Chang L-J, Tong J, Ginovart N, Wilson A, et al. Regional distribution of serotonin transporter protein in postmortem human brain: is the cerebellum a SERT-free brain region? Nuclear Medicine and Biology. 2005;32(2):123-8.

350. Samuvel DJ, Jayanthi LD, Bhat NR, Ramamoorthy S. A role for p38 mitogen-activated protein kinase in the regulation of the serotonin transporter: evidence for distinct cellular mechanisms involved in transporter surface expression. Journal of Neuroscience. 2005;25(1):29-41.

351. Horschitz S, Lau T, Schloss P. Glycine residues G338 and G342 are important determinants for serotonin transporter dimerisation and cell surface expression. Neurochemistry International. 2008;52(4-5):770-5.

352. Rasmussen TN, Plenge P, Bay T, Egebjerg J, Gether U. A single nucleotide polymorphism in the human serotonin transporter introduces a new site for N-linked glycosylation. Neuropharmacology. 2009;57(3):287-94.

353. Ahmed BA, Jeffus BC, Bukhari SI, Harney JT, Unal R, Lupashin VV, et al. Serotonin transamidates Rab4 and facilitates its binding to the C-terminus of SERT. Journal of Biological Chemistry. 2008.

354. Parthasarathy PT, Galam L, Huynh B, Yunus A, Abuelenen T, Castillo A, et al. MicroRNA 16 modulates epithelial sodium channel in human alveolar epithelial cells. Biochemical and Biophysical Research Communications. 2012;426(2):203-8.

355. Molitoris JK, McColl KS, Distelhorst CW. Glucocorticoid-mediated repression of the oncogenic microRNA cluster miR-17~ 92 contributes to the induction of Bim and initiation of apoptosis. Molecular Endocrinology. 2011;25(3):409-20.

356. Rainer J, Ploner C, Jesacher S, Ploner A, Eduardoff M, Mansha M, et al. Glucocorticoid-regulated microRNAs and mirtrons in acute lymphoblastic leukemia. Leukemia. 2009;23(4):746.

357. Hong Y, Wu J, Zhao J, Wang H, Liu Y, Chen T, et al. miR-29b and miR-29c are involved in Toll-like receptor control of glucocorticoid-induced apoptosis in human plasmacytoid dendritic cells. PLoS One. 2013;8(7):e69926.

358. De Iudicibus S, Lucafò M, Martelossi S, Pierobon C, Ventura A, Decorti G. MicroRNAs as tools to predict glucocorticoid response in inflammatory bowel diseases. World Journal of Gastroenterology: WJG. 2013;19(44):7947.

359. Bian Y, Qian W, Li H, Zhao RC, Shan WX, Weng X. Pathogenesis of glucocorticoid-induced avascular necrosis: A microarray analysis of gene expression in vitro. International Journal of Molecular Medicine. 2015;36(3):678-84.

360. Kawashima H, Numakawa T, Kumamaru E, Adachi N, Mizuno H, Ninomiya M, et al. Glucocorticoid attenuates brain-derived neurotrophic factor-dependent upregulation of glutamate receptors via the suppression of microRNA-132 expression. Neuroscience. 2010;165(4):1301-11.

361. Luo J, Wang Y, Lan D, Niu J, Miao J, Dong X, et al. Differential expression of serum microRNAs in glucocorticoid-resistant patients with ulcerative colitis. International Journal of Clinical and Experimental Pathology. 2018;11(2):936-46.

362. Johnson RF, Rennie N, Murphy V, Zakar T, Clifton V, Smith R. Expression of glucocorticoid receptor messenger ribonucleic acid transcripts in the human

placenta at term. The Journal of Clinical Endocrinology & Metabolism. 2008;93(12):4887-93.

363. Piek E, Heldin C-H, Ten Dijke P. Specificity, diversity, and regulation in TGF-β superfamily signaling. The FASEB Journal. 1999;13(15):2105-24.

364. Song C-Z, Tian X, Gelehrter TD. Glucocorticoid receptor inhibits transforming growth factor-β signaling by directly targeting the transcriptional activation function of Smad3. Proceedings of the National Academy of Sciences USA. 1999;96(21):11776-81.

365. Ben-David U, Siranosian B, Ha G, Tang H, Oren Y, Hinohara K, et al. Genetic and transcriptional evolution alters cancer cell line drug response. Nature. 2018;560(7718):325-30.