The Regulation of Multidrug Resistance Phosphoglycoprotein (MDR1/P-gp) and Breast Cancer Resistance Protein (BCRP) in the Human Placenta

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This thesis is submitted as a partial fulfillment of the M.Sc. program in Cellular and Molecular Medicine

January 2011

University of Ottawa

Ottawa, Ontario

ABSTRACT

Multidrug resistance phosphoglycoprotein (MDR1/P-gp) and breast cancer resistance protein (BCRP) were first isolated in chemoresistant cancer cells and have since been found in a variety of normal tissue, including the placenta. The potential function of MDR1/P-gp and BCRP in the human placenta is to protect the fetus from maternally circulating endogenous steroids and hormones, therapeutic drugs and toxins. Studies from our laboratory have shown that there is a significant decrease in MDR1/P-gp levels and an increase in BCRP levels in the syncytiotrophoblast of the human placenta with advancing gestation; however, the regulation of both proteins is not known. The objective of this study was to examine the role of maternal steroids in the regulation of MDR1/P-gp and BCRP in the human placenta. Term (38-40 weeks) placenta tissues were collected after scheduled, non-complicated cesarean sections at the Ottawa General Hospital and a modified version of the Kliman's technique was used to isolate trophoblast cells. Immunohistochemistry (IHC), western blot analysis and transport studies were used to determine the effect of maternal steroids on MDR1/P-gp and BCRP regulation. Maternal steroids (estrogen, progesterone and cortisol), present at high concentrations in maternal serum, did not have an effect on BCRP in human syncytiotrophoblast. Estrogen and progesterone did not alter MDR1/P-gp levels in human syncytiotrophoblast, but cortisol significantly decreased MDR1/P-gp levels. Dexamethasone, an agonist of the glucocorticoid receptor, did not have an effect on MDR1/P-gp levels. Furthermore, co-incubation with RU486, a synthetic glucocorticoid, did not reverse the inhibitory effect of cortisol on MDR1/P-gp levels. The decrease in MDR1/P-gp levels with advancing gestation could be due to the increase in cortisol levels in maternal circulation towards term. It would appear that cortisol is not acting via the glucocorticoid receptor to exert its regulatory effect on MDR1/P-gp.

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ABC	ATP-Binding Cassette
ABCB1	ATP-Binding Cassette, sub-family B
ABCC	ATP-Binding Cassette, sub-family C
ABCG2	ATP-Binding Cassette, sub-family G
ABCP	ABC transport in the placenta
ATCC	American Type Culture Collection
AZT	Zidovudine
BCRP	Breast Cancer Resistance Protein
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CAR	Constitutive androsterone receptor
DAB	Diaminobenzidine
DNAse	Deoxyribonuclease 1
DMEM	Dulbecco's modified eagles medium
ECL	Enhanced chemiluminescence
ERα	Estrogen receptor alpha
ERE	Estrogen response element
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
Gβ	G protein beta subunit
hCG	Human chorionic gonadotrophin

HIV	Human immunodeficiency virus
HRE	Hypoxia response elements
IHC	Immunohistochemistry
MDR1/P-gp	Multidrug Resistance Phosphoglycoprotein
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
MRP1-9	Multidrug Resistance-Associated Protein 1-9
MXR	Mitoxantrone resistance protein
NBD	Nucleotide binding domain
NCS	Newborn calf serum
PBS	Phosphate buffered saline
PhIP	Carcinogen 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine
PR	Progesterone receptor
PRA	Progesterone receptor A
PRB	Progesterone receptor B
PRE	Progesterone response element
PXR	Pregnane xenobiotic receptor
RU486	Mifepristone
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfate
TMD	Transmembrane domain

ACKNOWLEDGMENTS

This thesis is dedicated to my family, Eric, Sandy, Kelsey and Russell Rainey, for their undying support and love. I would especially like to thank my mother for her continued optimism, support, advice and tri-daily phone calls. I am eternally grateful.

I would like to thank my supervisor, Dr. William Gibb, for his guidance, encouragement, and above all his patience throughout my studies in his laboratory. I also appreciated his allowing me to take time to pursue volunteering opportunities, particularly Science Travels and The Olympic Winter Games in Vancouver, and being very supportive and enthusiastic about these endeavours. I am also grateful to Dr. Stephen Matthews at the University of Toronto, who is the collaborator and co-grant owner, for his advice regarding my project.

I wish to show my appreciation to my advisory committee, Dr. Gruslin, Dr. Cooke, and Dr. Tsang for their suggestions and providing me with direction regarding my project. Thank you for taking time out of your busy schedules to read my reports and come to my meetings.

Thank you to Meihua Sun, for her technical expertise and her advice in Dr. Gibb's lab. I would also like to thank Dorothy Yeboah, for teaching me laboratory techniques, especially for her patience in demonstrating human trophoblast cell culture.

I am indebted to Pauline Shields and Emma Webster for consenting tissues at the Ottawa General Hospital. My research was very much dependent on them and I am grateful.

Many thanks to my extended family, particularly my Aunt Nicole, Uncle John, and my Nama, for providing me with accommodations in Ottawa throughout my studies.

Finally, I would like to thank my fabulous friends for listening to me when I was stressed, for your endless encouragement and always picking me up when I'm down. I would especially like to thank Deana Conway and Danny Richardson for all their support in the past two and a half years.

CHAPTER 1: INTRODUCTION

1.1 ABC TRANSPORTERS

1.1.1 Overview

ATP-binding cassette (ABC) proteins are a large and functionally varied superfamily of proteins that use energy generated from ATP hydrolysis to move substrates across a membrane, usually against a concentration gradient (Dean, Hamon, & Chimini, 2001; Higgins, 1992; Childs & Ling, 1994; Dean & Allikmets, 1995). ABC transporters are present in all phyla and can either import or export substrates across cell membranes. In humans, these proteins export substrates from the cytoplasm or cell membrane to the exterior of the cell (Saurin, Hofnung, & Dassa, 1999). There are 49 known ABC proteins encoded by the human genome, which are divided into 7 subfamilies, A to G (Sheps *et al.*, 2004; Dean & Annilo, 2005). Three of these proteins are involved in drug transport in the human body; Multidrug Resistance Phosphoglycoprotein (MDR1/P-gp or ABCB1; encoded by the *MDR1* gene), Multidrug Resistance-Associated Protein 1-9 (MRP1-9; encoded by the *ABCC* gene), and Breast Cancer Resistance Protein (BCRP or ABCG2; encoded by the *BCRP* gene) (Litman *et al.*, 2001). The substrates of these proteins are diverse and overlapping, therefore it is thought that they collaborate to form a network to protect the body from xenobiotics (Sharom, 2008). ABC transporters can cause resistance to chemotherapy drugs in cancer cells and can also act as pharmacological barriers in normal tissue. MDR1/P-gp and BCRP will be the focus of this thesis.

1.1.2 MDR1/P-gp

Multidrug Resistance Phosphoglycorprotein, MDR1/P-gp, was first discovered by Juliano and Ling (1976). They found high levels of a 170 kDA protein on the membrane of Chinese hamster ovarian cells, which extruded colchicine out of the cells, causing drug resistance (Juliano & Ling, 1976). In 1987, the protein was named MDR1/P-gp (Ueda *et al.*, 1987).

MDR1/P-gp was initially studied in chemoresistant tumour cells (Juliano & Ling, 1976), but was later found in a variety of normal tissue, where the protein is localized at important barrier sites to limit the absorption of pharmacological agents and promote excretion of these substrates (Thiebaut *et al.*, 1987; Cordon-Cardo *et al.*, 1990). For example, on the intestinal epithelium, MDR1/P-gp extrudes substrates back into the gut lumen (Thiebaut *et al.*, 1987). The protein transports substrates out of the proximal tubule cells of the kidney into the urine and out of the liver into the bile (Schinkel & Jonker, 2003). MDR1/P-gp is also expressed at high levels in the blood-brain barrier and in the blood-testis barrier (Cordon-Cardo *et al.*, 1989). Our laboratory has shown MDR1/P-gp to be localized on the apical membrane of the syncytiotrophoblast of the placenta, where the protein transports substrates from the syncytiotrophoblast back into maternal blood, to limit substrate distribution to the fetus (Sun *et al.*, 2006).

MDR1/P-gp can efflux a large variety of structurally different substrates (Wang *et al.*, 2003). Substrates are typically, but not always, between 200 Da-1900 Da in size, have an aromatic ring in their structure and are uncharged or basic. However, these properties do not hold true for all substrates of the protein, as some are non-aromatic linear or circular in structure and acidic in pH

(Schinkel & Jonker, 2003). Pharmacological substrates of MDR1/P-gp include cardiac glycosides, antibiotics, and human immunodeficiency virus (HIV) protease inhibitors (Schinkel *et al.*, 1995; Schuetz *et al.*, 1996; Choo *et al.*, 2000). Furthermore, endogenous steroids such as cortisol (Ueda *et al.*, 1992) and synthetic steroids, such as dexamethasone and betamethasone, are also substrates of the protein (Meijer, Karssen, & de Kloet, 2003). Anticancer drugs that are unique substrates for MDR1/P-gp, but not BCRP or MRP, include cisplatin, taxols, and verapamil (Sharom, 2008). The large variety and number of substrates of MDR1/P-gp indicates a potential function of the protein in protecting the body from toxic xenobiotics, as well as a possible physiological function in the regulation of endogenous steroids levels.

One gene, *MDR1*, encodes MDR1/P-gp in humans (Chen *et al.*, 1986). Interestingly, two genes, *Mdr1a* and *Mdr1b*, encode two forms of the Mdr1/P-gp in the mouse. The two isoforms of the protein, Mdr1a/P-gp and Mdr1b/P-gp, have both unique and overlapping substrate specificities and localizations. For example, in the mouse, the heart, lungs, and kidney have similar levels of both *Mdr1a* and *Mdr1b* mRNA, however, the liver and brain have higher levels of *Mdr1a* mRNA and the placenta and uterus have higher levels of *Mdr1b* mRNA (Devault & Gros, 1990; Gros *et al.*, 1991; Kalabis *et al.*, 2005).

Homozygous *Mdr1a* knock-out mice, *Mdr1a* (-/-), were found to be healthy and fertile, and have normal lifespans. However, when the cages were sprayed with ivermectin, to treat a mite infestation, it was found that all *Mdr1a* (-/-) mice died, while the normal and heterozygous mice lived. Upon further investigation, it was found that there was 100-fold increase of invermectin in the brain, compared to wild-type mice (Schinkel *et al.*, 1994). This study indicated that Mdr1a/P-gp plays an important role in limiting entry of toxins into the brain.

1.1.3 BCRP

BCRP was originally discovered and cloned from the breast carcinoma cell line, MCF-7/AdrVp, by Doyle *et al.* (1998). The cell line was resistant to the chemotherapeutic agent doxorubicin, as well as daunorubicin and mitoxantrone (Doyle *et al.*, 1998). Shortly afterwards, two other groups independently cloned proteins that were almost identical to BCRP. ABCP (ABC transporter in the placenta) was isolated from the human placenta (Allikmets *et al.*, 1998) and MXR (mitoxantrone resistance protein) was isolated from S1-M1–80, a human colon cancer cell line that is resistant to mitoxantrone (Miyake *et al.*, 1999). Other than a few amino acid differences, BCRP, ABCP, and MXP are the same protein.

BCRP has been found in cancer cells and in normal tissue. Northern blot studies determined that BCRP mRNA was expressed at high levels in the human placenta, as well as being expressed in the liver, small intestine, brain, and testis (Doyle *et al.*, 1998). BCRP was localized to the apical membrane of syncytiotrophoblast of the placenta, the liver canalicular membrane, the apical membrane of the epithelium of the small intestine, the breast, and the blood-brain barrier using immunohistochemistry (IHC) (Maliepaard *et al.*, 2001). BCRP has also been proposed to provide protection against hypoxia via the efflux of toxic heme metabolites from the cytoplasm (Krishnamurthy *et al.*, 2004).

BCRP extrudes a broad range of xenobiotic and endogenous substrates, which are hydrophobic or hydrophilic in nature and can be either conjugated or unconjugated, including sulphate conjugates (Imai *et al.*, 2003). Many chemotherapeutic agents that BCRP transport are also substrates of MDR1/P-gp. For example, daunorubicin, doxorubicin and mitoxantrone are anti cancer drugs that are transported by both proteins (Doyle & Ross, 2003). However, the substrate specificity of BCRP only partially overlaps with MDR1/P-gp. Substrates unique to BCRP include flavopiridiol and SN-38, a topoisomerase 1 inhibitor (Mao & Unadkat, 2005).

BCRP also transports a variety of therapeutic drugs. Nitrofurantoin, an antibiotic, is a substrate of the protein (Merino *et al.*, 2005). Glyburide, an anti-diabetic drug, and the HIV drug zidovudine (AZT) are also pharmacological agents that are transported by BCRP (Gedeon *et al.*, 2006; X. Wang *et al.*, 2003). Cimetidine, a histamine H_2-receptor antagonist, was also found to be a substrate (Pavek *et al.*, 2005). Certain toxins are transported by BCRP, such as 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP), a carcinogen found in food and cigarette smoke (Pavek *et al.*, 2005). Furthermore, certain endogenous steroids are also known to be substrates of the protein, such as sulfo- conjugates of estrone or 17β-estradiol (Imai *et al.*, 2003). Interestingly, BCRP is not implicated in the transport of free estrogen (Pavek *et al.*, 2005). The protein is also known to transport folate, associating BCRP with folic acid homeostasis in cells (Hooijberg *et al.*, 2006). Considering the localization and substrate specificity of the protein, the potential function of BCRP is to protect the body from foreign BCRP substrates, such as toxins and pharmaceutical agents, and regulate endogenous BCRP substrates.

1.1.4 MDR1/P-gp and BCRP Protein Structure

The basic core structure of the human ABC protein consists of two hydrophilic nucleotide binding domains (NBDs), which are believed to bind and hydrolyze ATP, and two hydrophobic transmembrane domains (TMDs), which are thought to recognize and provide a translocation passageway for the substrate to cross the membrane (Higgins *et al.*, 1986). This is referred to as a full transporter and is typically organized TMD1-NBD1-TMD2-NBD2 (Loo & Clarke, 1999). Human ABC proteins can also be half transporters, containing one TMD and one NBD. The domain sequences of half transporters are NBD1-TMD1 or TMD1-NBD1 (Doyle *et al.*, 1998). In order to be functional, human ABC proteins require two TMDs and two NBDs; therefore, half transporters must form homo- or heterodimers to operate (Hyde *et al.*, 1990).

As previously stated, the *MDR1* gene encodes MDR1/P-gp in humans. The human protein is composed of 1280 amino acids; with molecular mass of approximately 170 kDa (Juliano & Ling, 1976; Hyde *et al.*, 1990), although some publications have suggested a molecular mass of 140-170 kDa (Ling *et al.*, 1983). MDR1/P-gp is a full transporter composed of two NBDs and two TMDs, with 6 α-helices per TMD (**Figure 1**) (Schinkel & Jonker, 2003; Borst & Elferink, 2002).

The *BCRP* gene encodes BCRP in humans. BCRP is 72 kDa in size and composed of 665 amino acids (Doyle *et al.*, 1998). The configuration of BCRP is that of a half-transporter, NBD1-TMD1 (**Figure 2**); therefore the protein is thought to homodimerize or forms a complex to become functional (Ozvegy, Varadi, & Sarkadi, 2002). It has been suggested that BCRP forms a homodimer through S-S bonds. This disulphide bond bridge has been considered necessary for drug transport function, due to the fact that function was abolished by a dominant negative mutant (Kage *et al.*, 2002). Yeboah *et al.* (2006b) observed a protein of approximately 200 kDa, under non-reducing conditions in human placental tissue. These results indicate that BCRP may function as a multimer, instead of a homodimer, in the human placenta (Yeboah *et al.* 2006b).

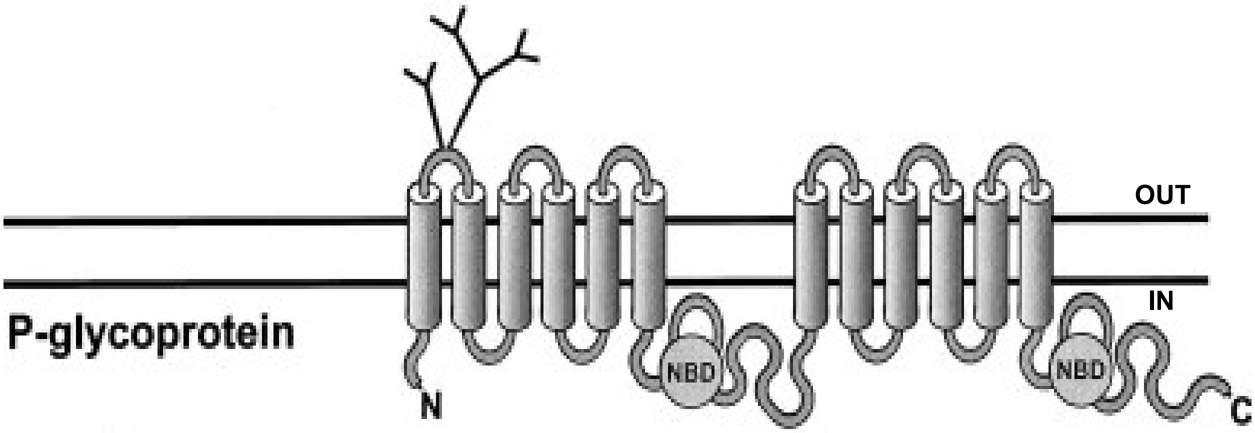

Figure 1| Proposed structure of MDR1/P-gp. MDR1/P-gp is composed of two transmembrane domains (TMDs), with 6 transmembrane α-helices each, and two NBDs. MDR1/P-gp is considered a full transporter. Modified from Shinkel and Jonker (2003).

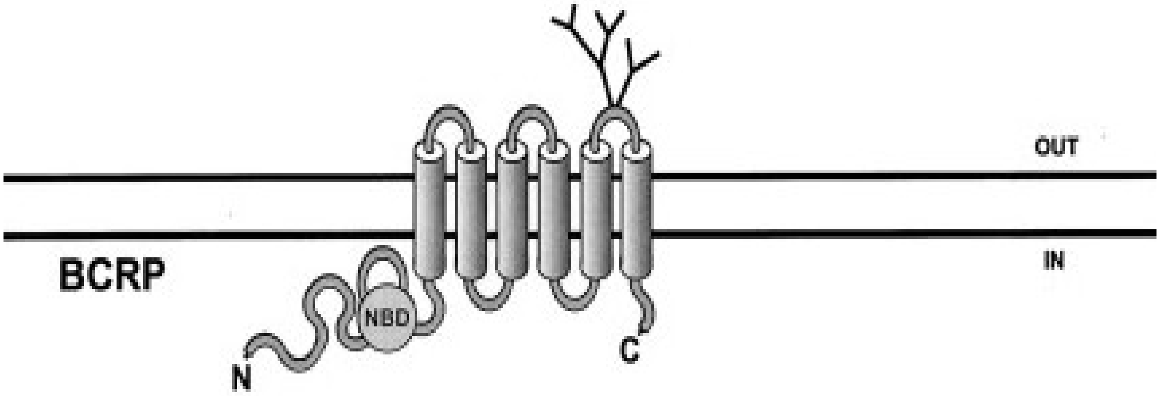

Figure 2| Proposed structure of BCRP. BCRP is composed of one NBD and one TMD. BCRP is considered a half transporter. Adapted from Schinkel and Jonker (2003).

1.1.5 Regulation of Human MDR1/P-gp and BCRP

The human *MDR1* gene promoter has been characterized; however transcriptional regulation is still not entirely understood. The *MDR1* gene promoter is TATA-less, therefore, it has multiple start sites (Labialle *et al.*, 2002), with transcription potentially beginning upstream of a major start site (Ueda, Pastan, & Gottesman, 1987). The promoter contains a GC-box, a GC-rich region where the transcription factor Sp1 binds (Cornwell & Smith, 1993), and a CCAAT, or Y-box, where the protein NF-Y has been shown to bind in several studies (Jin & Scotto, 1998; Sundseth *et al.*, 1997). It is thought that the GC-box and the Y-box may work together to regulate *MDR1* gene transcription (Roder *et al.*, 1999). The promoter of the *MDR1* gene also contains an AP-1 element, where c-Jun and c-Fos proteins may be involved in the complex (Daschner *et al.*, 1999). Furthermore, in the mouse a putative glucocorticoid response element (GRE) has been characterized in the *Mdr1b* gene promoter. This indicates that glucocorticoids could potentially regulate MDR1 gene transcription (Cohen *et al.*, 1991). It remains to be determined whether the human *MDR1* gene promoter contains a GRE. MDR1/P-gp can also be regulated by mRNA stability or post-translational processing (Schinkel *et al.*, 1993).

The human *BCRP* gene extents over 66 kb and has 16 exons and 15 introns. Similar to the *MDR1* gene promoter, the promoter is TATA-less. Other characteristics of the *BCRP* promoter include several Sp1 sites (GC boxes), AP-1 and AP-2 binding sites, a CCAATT box and a putative CpG island (Bailey-Dell *et al.*, 2001). Interestingly, the *BCRP* promoter may contain one or more hypoxia response elements (HRE) (Krishnamurthy *et al.*, 2004), indicating that BCRP has the potential to be transcriptionally regulated by hypoxic conditions. An estrogen response element (ERE) (Ee *et al.*, 2004) and a progesterone response element (PRE) (Wang *et* *al.*, 2008) have also been identified upstream of the transcription start site and they have been shown to be functional. Consequently, sex steroids, such as estrogen and progesterone, have been suggested as possible regulators of BCRP.

The regulation of *MDR1* and *BCRP* still remains unclear.

1.2 The Human Placenta

1.2.1 Overview

The human placenta plays an integral role in pregnancy and the healthy development of the fetus. It acts as the pulmonary, renal, and hepatic system of the fetus in utero. The placenta brings the blood of mother and fetus in close proximity, to exchange gas, ions, and nutrients from mother to fetus and to remove toxic waste products from fetal circulation (Ceckova-Novotna, Pavek, & Staud, 2006). Furthermore, the placenta is known to possess drug metabolizing enzymes involved in biotransformation and detoxification reactions and drug transporters that influence the absorption, distribution and metabolism of xenobiotics (Pasanen, 1999; Syme, Paxton, & Keelan, 2004).

An important example of substances traversing the placenta was thalidomide, which was prescribed to pregnant women as a sedative drug in the 1960s. Thalidomide produced teratogenicity, such as skeletal malformation of the limbs, when given at certain time points during pregnancy (Botting, 2002). Following this discovery, the idea that the placenta was impermeable was disproven and it has now been shown that most pharmaceutical agents that are administered to the mother during pregnancy will cross the placenta, to some extent, and reach the fetus (Syme *et al.*, 2004; Pacifici & Nottoli, 1995; Audus, 1999a).

1.2.2 Structure

The feto-placenta-maternal circulation does not develop until approximately the 10th week of pregnancy, after organogenesis of the embryo. Therefore, during this time, xenobiotics that are present in maternal circulation can only reach the embryo by way of diffusion through extracellular fluid (Syme *et al.*, 2004). After the 10th week of pregnancy, maternal blood enters the intervillous space, through spiral arteries in the decidua basalis. In the intervillous space maternal blood comes in contact with syncytiotrophoblast, a multinucleated barrier composed of fusion of the underlying cytotrophoblast cells. The syncytiotrophoblast is polarized epithelial-like layer, with an apical, brush-bordered membrane that is in contact with maternal blood and a basolateral membrane that is in contact with cytotrophoblast, stroma cells, or endothelial cells of the fetal blood vessels (Young, Allen, & Audus, 2003). The syncytiotrophoblast layer is the rate-limiting barrier for substances crossing the placenta. Oxygen, nutrients, and xenobiotics from maternal blood first cross the syncytiotrophoblast, then cytotrophoblast and finally the endothelial cells of fetal blood vessels into the fetal blood (Leslie, Deeley, & Cole, 2005). The placenta and the developing fetus are attached by the umbilical cord, containing one umbilical vein and two umbilical arteries (**Figure 3A**). Oxygen rich blood, carrying nutrients and possibly xenobiotics, is transported to the fetus by the umbilical vein. Deoxygenated blood carries waste products through two umbilical arteries, to reach maternal blood through the reverse pathway (Ceckova-Novotna *et al.*, 2006).

Both MDR1/P-gp and BCRP are expressed on the apical membrane of syncytiotrophoblast in the human placenta, to efflux substrates back into maternal blood and prevent distribution to the fetus (**Figure 3C**) (MacFarland *et al.*, 1994; Doyle *et al.*, 1998).

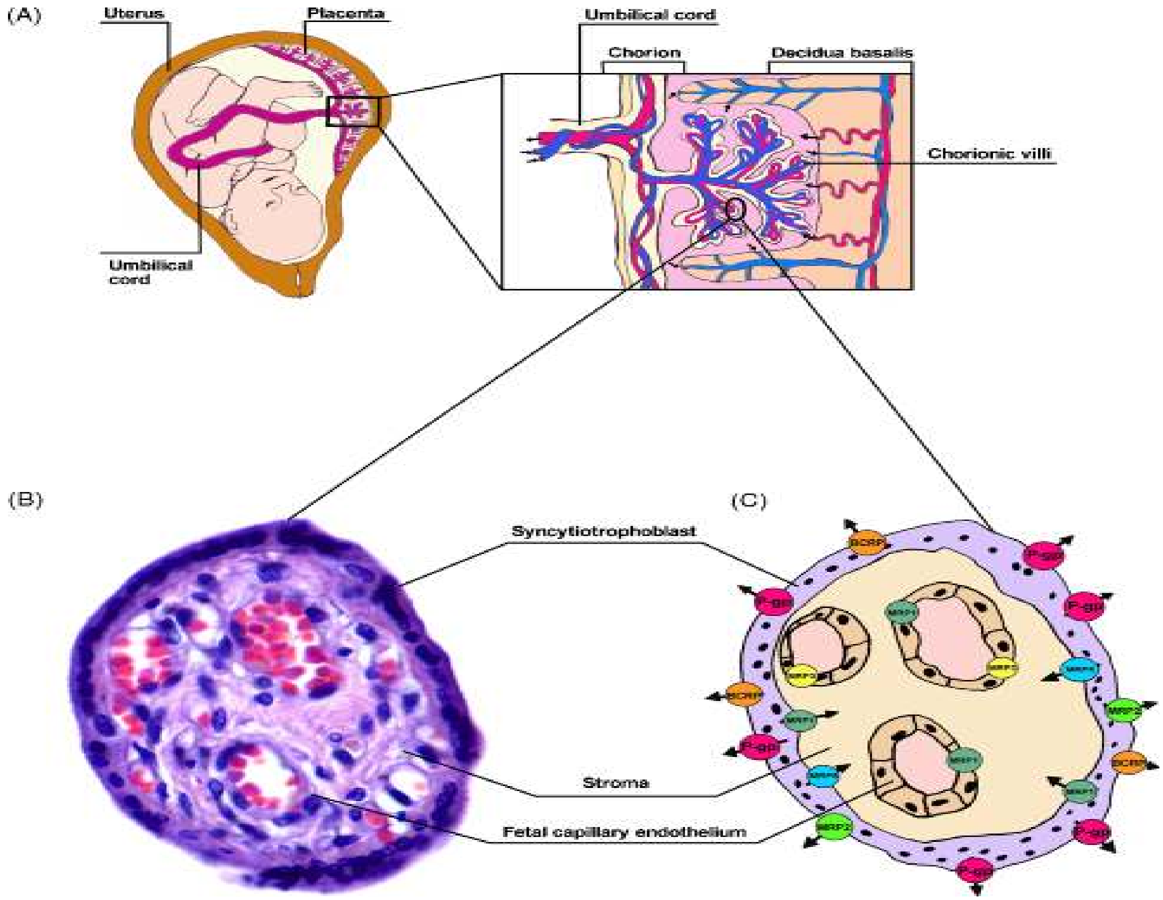

Figure 3| The structure of the human placenta. (A) A cross section of the fetus at term, inside the uterus of the mother, as well as a cross section of the placenta. (B) Hematoxylin–eosin stained paraffin sections of terminal villi. (C) A proposed model of drug transporters on syncytiotrophoblast and fetal blood vessels. Orange: BCRP, pink: MDR1/P-gp. Adopted from Ceckova-Novotna et al. (2006).

1.3 ABC Transporters in the Human Placenta

1.3.1 Maternal Pharmacotherapy

Women can be treated for a variety of pre-existing or acquired conditions throughout pregnancy. For example, hypertension, diabetes, and depression are disease states for which it is crucial for pregnant women to take medication. In one study, 96% of 578 women interviewed reported taking a prescription drug during pregnancy. The same study reported that 93% of pregnant women had taken over-the-counter medication (Glover *et al.*, 2003). These alarmingly high statistics suggest that a majority of women obtain prescription and over-the-counter medication during pregnancy. In addition, it has also been estimated that 5-10% of pregnant women have taken Food and Drug Administration (FDA) drugs classified as category D or category X. Category D include those drugs where there is evidence of fetal risk but the benefits may outweigh the risks, and category X, such as warfarin derivatives and benzodiazepines sedatives, include drugs where there is fetal teratogenic evidence and the risk of taking the drug clearly outweighs any benefits (Andrade *et al.*, 2004; Andrade *et al.*, 2006; Cooper, Hickson, & Ray, 2004). The health of both the mother and fetus must be considered independently and the health benefits must outweigh the potential risk of toxicity to the fetus (Gedeon & Koren, 2006). Transplacental transfer of potentially harmful substances cannot be studied *in vivo* in pregnant women, due to ethical, moral and legal restrictions. Furthermore, clinical trials are infrequently carried out in pregnant women due to the same reasons (Behravan & Piquette-Miller, 2007). On account of this, animal models and *in vitro* studies are often used to study drug transmission across the placenta.

There are multiple ways in which maternally administered drugs can cross the placenta from maternal blood to the fetus, or vice versa, which include passive diffusion, facilitated diffusion, and active transport. For the most part, drugs cross the placenta from maternal circulation via passive diffusion (Audus, 1999b). The major determinants for transplacental passive diffusion of drugs is molecular weight (>500 Da), lipophilicity, ionization, and protein binding (Pacifici & Nottoli, 1995; Syme *et al.*, 2004). In opposition to passive diffusion, ABC transporters in the syncytiotrophoblast were identified to participate in active transport of substrates out of the placenta. MDR1/P-gp was the first ABC transporter to be found in the human placenta (Cordon-Cardo *et al.*, 1990). BCRP is also expressed at high levels and therefore it may play an important role in fetal protection (Doyle *et al.*, 1998; Allikmets *et al.*, 1998). Studies in the human placenta are limited, however *in vitro* and *in vivo* studies in animals have helped to identify the protective role that MDR1/P-gp and BCRP play in pregnancy.

1.3.2 MDRI/P-gp in the Human Placenta

Animal models have shown the importance of MDR1/P-gp in the placenta. CF-1 mice, in which 25% of the population have spontaneous mutations in the *Mdr1a* gene, can be divided into -/- homozygous negative (phenotypically comparable to Mdr1a knockout mice), +/- heterozygous, or +/+ homozygous positive for Mdr1a. Fetuses with genotype Mdr1a (-/-) had 100% occurrence of cleft palate, when exposed to avermectin, used for parasite control in veterinary medicine. Heterozygous fetuses were less sensitive and homozygous positive had no adverse effects when exposed to the toxin (Lankas *et al.*, 1998). Even though Mdr1b/P-gp is the main isoform in the placenta, this indicates a function of Mdr1a/P-gp in protecting the body from toxic xenobiotics.

Our laboratory localized of MDR1/P-gp on the apical membrane of the syncytiotrophoblasts in the human placenta and examined its level in human placental tissue throughout gestation. We have shown that there is a significant decrease in MDR1/P-gp levels in the syncytiotrophoblast of the human placenta with increasing gestation. Levels of MDR1/P-gp are high in mid-gestation, but dramatically decrease at term (Sun *et al.*, 2006). Similar results have been found by our group in the mouse (Kalabis *et al.*, 2005) and this decrease in MDR1/P-gp correlated with an increase in [^{3}H]digoxin, a MDR1/P-gp substrate, crossing the placenta (Petropoulos *et al.*, 2007). MDR1/P-gp may protect the developing fetus from high levels of maternal steroids, maternally administered drugs and environmental toxins and could be one of the primary means of fetal protection (**Figure 4**). The dramatic decrease in the level of this efflux protein in human placenta towards term can subject the term fetus to these substances in the maternal circulation.

1.3.3 BCRP in the Placenta

Animal models have also shown the importance of BCRP in the placenta. For example, glyburide, a substrate of BCRP, was injected in wild-type and Bcrp -/- pregnant mice. Both groups were compared at different time point after glyburide injection. Glyburide concentration in the maternal plasma samples of both wild-type and Bcrp -/- pregnant mice were comparable. However, glyburide concentrations in the fetal tissue homogenate of Bcrp knock-out mice were two times greater than the concentration in the fetal tissue homogenate of the wild-type group (Zhou *et al.*, 2008). One of the potential functions of BCRP is to limit the entry of pharmaceutical agents into fetal circulation.

BCRP is present at high levels in the human placenta (Doyle *et al.*, 1998). Our laboratory has localized BCRP to the apical membrane of the syncytiotrophoblasts. Furthermore, we have shown that BCRP levels increase at term, compared to earlier time points of gestation (Yeboah *et al.*, 2006a). This increase in protein levels at term may provide enhanced fetal protection towards the end of pregnancy. Similar to MDR1/P-gp, the potential function of BCRP in the human placenta is to increase fetal protection from pharmaceuticals, toxins, and endogenous steroids in maternal circulation (**Figure 4**).

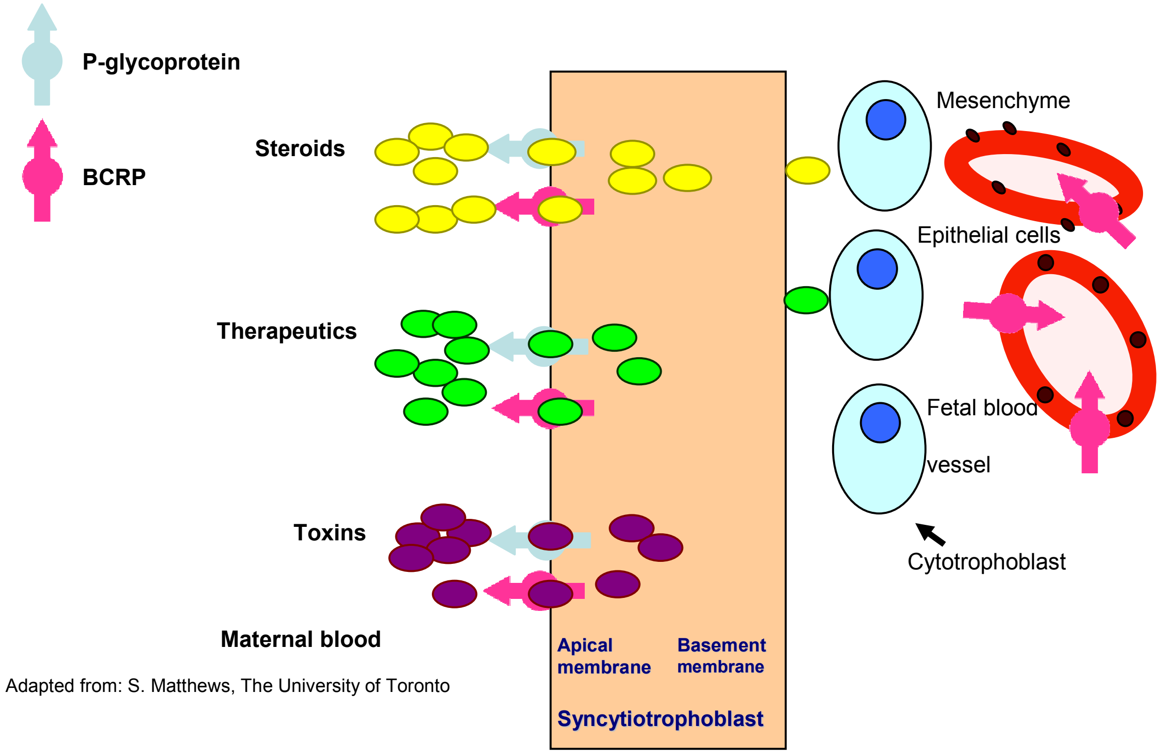

Figure 4| The potential function of MDR1/P-gp and BCRP in the human placenta. Adapted from Dr S. Matthews, The University of Toronto, 2011.

1.3.4 Regulation of MDR1/P-gp and BCRP by Sex Steroids

There is little known about the physiological regulation of MDR1/P-gp and BCRP in the human placenta. Estrogen and progesterone levels in maternal serum increase throughout pregnancy (H. Wang *et al.*, 2006), coinciding with the decrease in MDR1/P-gp. Estrogen has been shown to regulate MDR1/P-gp in human cell lines, although the results are conflicting (Biing *et al.*, 1994) (Kim & Benet, 2004; Zampieri *et al.*, 2002; Mutoh *et al.*, 2006). Furthermore, one study has implicated estrogen in upregulating MDR1/P-gp in human trophoblast cell culture (Evseenko, Paxton, & Keelan, 2007). However, in this study only one concentration of the steroid was used. Previous studies have shown that progesterone regulated MDR1/P-gp levels in cell lines (Mallick & Horwitz, 1997; Piekarz, Cohen, & Horwitz, 1993), the mouse (Yang *et al.*, 1989), and human trophoblast cell culture (Evseenko *et al.*, 2007), however Petropoulos *et al.*(2007) found that progesterone did not have an effect on Mdr1/P-gp in the mouse placenta (Petropoulos *et al.*, 2007). In combination, progesterone and estrogen has been shown to increase Mdr1/P-gp in the secretory epithelium of the uterus of the mouse in early pregnancy (Arceci *et al.*, 1990; Yang *et al.*, 1989). Although previous studies are conflicting, estrogen and progesterone may be potential regulators of MDR1/P-gp in the human placenta, alone or in combination.

Studies regarding the regulation of placental BCRP during pregnancy are extremely limited. The increase in estrogen and progesterone during pregnancy parallels the increase in BCRP, which may implicate these steroids in the protein's regulation. Previous studies have shown that estrogen regulated BCRP in human cell lines (Imai *et al.*, 2005; H. Wang *et al.*, 2006; Zhang *et al.*, 2006) and human trophoblast cell culture (Evseenko *et al.*, 2007), however, again, results are contradictory. Progesterone up-regulated BCRP in the human choricarcinoma BeWo cell line (H. Wang *et al.*, 2008), but had no effect on BCRP in the mouse placenta or human trophoblast cell culture (Kalabis *et al.*, 2007; Evseenko *et al.*, 2007). Due to conflicting regulatory effects of estrogen and progesterone on BCRP in previous studies, these steroids must be further investigated in human trophoblast culture.

Endogenous cortisol levels also increase in late gestation and are believed to be involved in the onset of labour (Fowden, Li, & Forhead, 1998), coinciding with the decrease in MDR1/P-gp and an increase in BCRP. In addition, pregnant women could potentially be exposed to synthetic glucocorticoids, such as dexamethasone, which are given to approximately 10% of mothers who are at risk of preterm pregnancy to aid in fetal lung development (Koenen *et al.*, 2007). Studies regarding glucocorticoid regulation of MDR1/P-gp and BCRP are extremely limited. In regards to MDR1/P-gp, in the mouse placenta, dexamethasone increased the protein in late pregnancy (Petropoulos, Gibb, & Matthews, 2010). A few studies have implicated dexamethasone in the down-regulation of BCRP in breast cancer cell lines (Elahian, Kalalinia, & Behravan, 2009; Elahian, Kalalinia, & Behravan, 2010; Honorat *et al.*, 2008), however, there are no studies in which glucocorticoid regulation of BCRP has been investigated in the placenta. Glucocorticoid regulation of ABC proteins, MDR1/P-gp and BCRP, in the placenta is a subject that has not been thoroughly investigated and warrants further study.

1.4 Choriocarcinoma Cell Lines

1.4.1 BeWo Cell Lines

Pattillo and Gey (1968) established the BeWo cell line from a human carcinoma of the placenta. This cell line is an extensively used model for the study of trophoblast cell function in vitro, due to the fact that BeWo cells show comparable morphological characteristics, such as multiple nuclei per cytoplasm and "brush border" appearance. Biochemical markers in the cell line, for instance the production of human chorionic gonadotrophin (hCG) and placental specific proteins SP1 and placental lactogen (Wice *et al.*, 1990), are also similar to human trophoblast cells. Furthermore, BeWo cells produce hormones, such as progesterone, that are also produced by syncytiotrophoblast of the human placenta *in vivo* (Pattillo & Gey, 1968; Prouillac *et al.*, 2009). The BeWo cell line is also appealing for *in vitro* use because it is consistent, maintained without difficulty, and grows quickly to confluency when plated in culture (Liu, Soares, & Audus, 1997).

It is controversial within the literature whether BeWo spontaneously syncytialize in culture, without the aid of agents which increase intracellular levels of cyclic adenosine monophosphate (cAMP), for example, forskolin. The majority of publications agree that spontaneous fusion of BeWo cells in culture is low but syncytialization is augmented by forskolin. For example, Borges *et al.* (2003) used fluorescence microscopy to study the syncytialization of BeWo cells in culture. The BeWo cells were divided into two separate groups, one group injected with cytoplasmic green-fluorescent protein and the other with cytoplasmic red-fluorescent protein. Fluorescence microscopy was used to determine if BeWo cells syncytialize, thus mixing the red and green colours. Untreated BeWo cells fusion index was less the percent, calculated by the number of nuclei in the syncytia/total number of nuclei x 100. However, when BeWo cells were treated with forskolin for 48 hours, the fusion index increased to 11 percent (Borges *et al.*, 2003). Rote *et al.* (2005) have repeatedly observed that 5-10% of BeWo cells spontaneously fused in media alone, whereas 70-80% of BeWo cells syncytialized after 24 hours treatment with forksolin (Rote, 2005). Similarly, Orendi *et al.* (2010) compared spontaneous syncytialization of BeWo cells to syncytialization when treated with forskolin. Quantification of immunofluoresence staining for biomarker protein of syncytialization, the β subunit of hCG, showed that BeWo cells had a spontaneous fusion rate of 4.9% and treatment with forskolin raised this rate to 50.5% (Orendi *et al.*, 2010). However, some research groups have questioned whether BeWo cells spontaneously fuse (Evseenko, Paxton, & Keelan, 2006a; Coutifaris *et al.*, 1991). Coutifaris *et al.* (1991) concluded that BeWo cells "aggregate" under standard settings, but do not syncytialize unless cAMP analogues or substances which increase cAMP levels are administered (Coutifaris *et al.*, 1991).

1.4.2 ABC Transporters in the BeWo Cell Line

MDR1/P-gp is found in the placenta; however, it is controversial in the literature whether MDR1/P-gp is present in BeWo cells. Magnarin *et al.* (2008) could not detect *MDR1* mRNA expression and protein in the BeWo cell line; using RT-PCR and Western blot analysis respectively (Magnarin *et al.*, 2008). Low levels of MDR1/P-gp were found in the BeWo cell line by other groups (Atkinson *et al.*, 2003; Evseenko, Paxton, & Keelan, 2006a). Ushigome *et al.* (2000) found MDR1/P-gp, using Western blot analysis in the BeWo cell line (Ushigome *et al.*, 2000). Furthermore, the efflux of tritiated substrates (vinblastine, vincristine, and digoxin) of the protein was decreased by inhibitors of MDR1/P-gp, including cyclosporine A and verapamil, in BeWo cells (Ushigome *et al.*, 2000). Utoguchi *et al.* (2000) also found MDR1/P-gp in the BeWo cell line using Western analysis (Utoguchi *et al.*, 2000). Magnarin et al. (2008) suggested that the differences between the findings may be due to differences in conditions of cell cultures (Magnarin *et al.*, 2008). Interestingly, Mark and Waddell (2006) observed an increase level of MDR1/P-gp when BeWo cells syncytialized (Mark & Waddell, 2006).

High levels of endogenous BCRP in the BeWo cell line have been repeatedly observed at the mRNA (Ceckova *et al.*, 2006; Evseenko, Paxton, & Keelan, 2006b) and protein level (Bailey-Dell *et al.*, 2001; Ceckova *et al.*, 2006; Evseenko, Paxton, & Keelan, 2006a). Evseenko, Paxton and Keelan (2006) found significantly higher levels of BCRP mRNA and protein in the BeWo cell line, compared to cultured human trophoblast cells. Furthermore, BeWo cells showed functionally active BCRP, similar to that found in cultured trophoblast cells, using the Hoechst 33342 fluorescence assay.

1.5 RATIONALE

Pregnant women may require medication during pregnancy for a variety of conditions, for example hypertension, diabetes or even cancer. An alarmingly high number of women have reported taking prescription or over the counter medications throughout pregnancy and furthermore, synthetic glucocorticoids are given to approximately 10% of mothers who threaten preterm delivery.

ABC transporters, such as MDR1/P-gp and BCRP, are known to actively transport substrates out of the placenta, potentially functioning to protect the fetus from maternally circulating endogenous steroids, drugs and toxins. Understanding how MDR1/P-gp and BCRP are regulated in the human placenta will enable the regulation of fetal protection against maternally circulating therapeutic agents and toxins, develop treatment options for mother and fetus, and increase fetal protection in pathological pregnancies. Progesterone, estrogen and glucocorticoids are present at high levels in maternal plasma and have been shown to regulate MDR1/P-gp and BCRP in other systems.

1.6 PURPOSE

The purpose of this study was to examine the role of steroids in the regulation of ABC transporters, MDR1/P-gp and BCRP, in the human placenta.

1.7 HYPOTHESES

1) Steroids are responsible for the decrease in MDR1/P-gp levels that occur in the human placenta at term.
2) Steroids are responsible for the increase in BCRP levels that occur in the human placenta at term.

1.8 OBJECTIVES

Objective 1: To determine the effects of steroids on the regulation of MDR1/P-gp levels in the human placenta.

1) Localize MDR1/P-gp in human placental tissue.
2) Investigate the presence of MDR1/P-gp levels in human placental tissue and trophoblast primary cell culture.
3) Determine the effect of progesterone and estrogen, alone and in combination, on MDR1/P-gp.
4) Examine the regulatory effects of glucocorticoids on MDR1/P-gp

Objective 2: To determine the effects of steroids on the regulation of BCRP levels in the human placenta.

1) Investigate the presence of BCRP levels in human placental tissue.
2) Examine the effects of estrogen and progesterone on BCRP.
3) Examine the regulatory effects of glucocorticoids on BCRP.

Objective 3: To examine MDR1/P-gp and BCRP in the BeWo cells line as a potential model for human trophoblasts.

1) Investigate MDR1/P-gp and BCRP in the BeWo cell line.

2) Examine the effect forskolin on syncytialization of BeWo cells in culture and MDR1/P-gp and BCRP levels.

CHAPTER 2: MATERIALS AND METHODS

2.1 Tissue Collection

Term (38-40 weeks) placenta tissue was used as a source of trophoblast cells. Placenta tissues, with no indication of infection, were collected after scheduled, non-complicated cesarean sections at the Ottawa General Hospital, preceding labour. Approval was obtained from the ethics committee.

2.2 Trophoblast Primary Cell Culture

A modified (Premyslova *et al.*, 2003) version of the Kliman's method (Kliman *et al.*, 1986) was used to isolate trophoblast cells. In short, 60 g of syncytiotrophoblast tissue was rinsed with 0.9% NaCl (EM Science, NJ, USA). The tissue was coarsely minced and transferred to Dulbecco's modified eagles medium, DMEM (Wisent Inc, QC, Canada), with 0.125% trypsin and 0.02% deoxyribonuclease 1 (DNase; Sigma-Aldrich MO, USA), and shaken in a 37^{O}C water bath for three 30 minute digestions. Newborn calf serum, NCS (Wisent) was used to stop enzyme activity. Cells pooled from the digests were layered over a 5-70% Percoll (Sigma) gradient and centrifuged for 20 minutes at 2500xg. Trophoblast cells were isolated between density markers of 1.049 g/l and 1.06 g/l. Isolated cells were cultured in a DMEM culture medium supplemented with 10% fetal bovine serum, FBS (Wisent), and 1% antibiotic-antimycotic solution (Sigma). 2.5 million cells/mL were plated on a six well plate, 2.5 mL per well, for western analysis. 1.3 million cells/mL were plated on an eight-well chamber slides, 3.2 mL per slide, for IHC, and 1 million cells/mL were plated on a 24 well plate, 1 mL per well, for functional transport assay. The cells were incubated at 37^{O}C, with 5% $CO_{2,}$ for 96 hours to allow complete syncytialization. Culture medium was replaced every 48 hours. For steroid studies, cortisol (500 nM-5μM; Sigma), dexamethasone (500 nM-5μM; Sigma), estrogen (500 nM-5μM; Sigma), and progesterone (500 nM-5μM; Sigma) were introduced into culture medium after 96 hours. Protein was isolated and functional activity was assessed after 24 hours.

2.3 BeWo Cell Culture

The BeWo cell line was obtained from American Type Culture Collection (ATCC). BeWo cells were maintained in 75 cm^2 flasks, in F-12K medium, Kaighn's Modification of Ham's F-12 medium (ATCC, Manassas,VA; #CCL-98), supplemented with 10% FBS and 1% antibiotic-antimycotic solution. The cells were incubated at $37^{O}C$, with 5% CO_2. At 70% confluence, cells were plated in six-well plates for western analysis or eight-well chamber slides for IHC. BeWo cells were seeded in six-well plates for 24 hours, after which forkolin (20 μM; Sigma) was added to the F-12K medium. Protein was isolated after 72 hours of forskolin treatment.

2.4 Immunohistochemistry

Cells were fixed onto slides with 10% buffered formalin phosphate (Fisher Scientific, NJ, USA), after being washed twice with PBS, and dehydrated in 75% ethanol before being stored in 95% ethanol at 4 ^{O}C. IHC methods were executed as previously described (Sun *et al.*, 2006; Yeboah *et al.*, 2006a). In short, slides were taken out of storage and rehydrated in 75% and 50% ethanol, for 5 minutes respectively. Slides were washed twice in phosphate buffered saline (PBS; 5 minutes/wash), before natural endogenous peroxidase activity was blocked with H_2O_2 (BDH

Canada) in methanol, for 30 minutes. Non-specific binding was obstructed by incubation with PBS containing 1.5% normal horse serum (Vector Laboratories, CA, USA), for 20 minutes, after slides were washed twice in PBS (10 minutes/wash). Slides were incubated with cytokeratin (1:3000; Dako CA, USA), vimentin (1:3000; DakoCytomation, Denmark), antidesmosomal cytokeratin (1:500-1000; Sigma), or D-11 (1:500; Santa-Cruz Biotechnology, CA, USA), summarized in **Table 1**, and washed twice in PBS (5 minutes/wash). Rabbit anti mouse biotinylated antibody (Vector Laboratories) was used to incubate slides for 30 minutes. The avidin-biotin-peroxidase technique, from the Vectastain Elite ABC kit (Vector Laboratories), was used to visualize protein, using diaminobenzidine, DAB (Sigma), as a substrate. Haemotoxylin (EMD), which stained the nucleus and washed off non-specific staining, was used as a counterstain. Slides were dehydrated in serial dilutions of ethanol, cleared in xylene (EM Science), and mounted in permount (Fisher Scientific).

Primary Antibody		Secondary Antibody	
Name	Dilution	Name	Dilution
Cytokeratin (Dako)	1:3000, 2% BSA	Anti- Rabbit IgG biotinylated antibody (Vector)	3 drops/10 mL PBS, 2% BSA
D-11 (Santa Cruz)	1:500, 2% BSA	Anti- Mouse IgG biotinylated antibody (Vector)	3 drops/10 mL PBS, 2% BSA
Antidesmosomal Cytokeratin (Sigma)	1:3000, 2% BSA	Anti-Mouse IgG biotinylated antibody (Vector)	3 drops/10 mL PBS, 2% BSA
Vimentin (DakoCytomation)	1:3000, 2% BSA	Anti-Mouse IgG biotinylated antibody (Vector)	3 drops/10 mL PBS, 2% BSA

Table 1: Immunohistochemistry antibodies and dilutions. BSA: bovine serum albumin

2.5 Western blotting

PBS, containing 13.7mM NaCl, 8mM Na_2HPO_4 (Sigma), 2.67mM KCl (Sigma), and 1.48mM KH_2PO_4 (Aldrich WS, USA), was used to wash cells, twice. Cells were scraped in 1mL of cold PBS, collected, and centrifuged at 18500 xg. The supernatant was discarded and the pellet was resuspended in 50 μL of cell lysis buffer, containing PBS, 1% Nonidet P-40 (Sigma), 0.1% sodium dodecyl sulfate (SDS; Sigma), 0.5% sodium deoxycholate (Sigma), and Pefabloc SC Plus inhibitors (Roche Molecular Biochemicals; Dorval, Quebec). The samples were sonicated and then centrifuged at 25 200 xg, at 4 ^{O}C, for 20 minutes. The protein containing supernatant was collected, and protein concentration was determined using the Bradford assay (Bio-Rad, Richmond, CA, USA). Samples were diluted at a 1:1 ratio in Laemmli sample buffer (Bio-Rad), with β-mercaptoethanol (Bio-Rad) added as a reducing agent (1:50). Protein (40 μg) was separated on 4-15% polyacrylamide Ready Gel (Bio-Rad) and transferred to a nitrocellulose membrane (Bio-Rad) at 100V for 1 hour. First, the membrane was blocked for 4 hours with 5% non-fat dry milk (Bio-Rad) dissolved in a mixture PBS and 0.05% tween (Bio-Rad). The membrane was then incubated overnight, at 4 ^{O}C, with a mouse monoclonal anti human MDR1 antibody, D-11 (1:200; Santa-Cruz), in 2% non-fat dry milk, preceded by incubation with a horse peroxidase linked anti-mouse secondary antibody (1:1000; GE Healthcare, UK) in 2% non-fat dry milk, for 1 hour. The enhanced chemiluminescence (ECL) system (GE Healthcare) was used to detect proteins on Kodak BioMax MR-1 film (Kodak) and densitometry was used to quantify bands. G beta (Gβ; Santa-Cruz Biotechnology) was used as an internal control (1:1000). The membrane was then stripped for 30 minutes with Western Blot Stripping Buffer (ThermoFisher Scientific, NJ, USA), and blocked overnight with 5% non-fat dry milk dissolved in a mixture PBS and 0.05% Tween. This was followed by incubation with a rabbit polyclonal anti human

BCRP antibody, H-70 (1:200; Santa-Cruz Biotechnology) or BXP-21 (1:200; Santa Cruz) in 1% bovine serum albumin (BSA), overnight at 4 ^{O}C. The membrane was then incubated with horse radish peroxidase linked anti-rabbit secondary antibody (1:1000; GE Healthcare), for 1 hour, and bands were quantified using densitometry with Gβ. A list of primary and secondary antibodies and their respective dilutions are provided in **Table 2**.

Primary Antibody		Secondary Antibody	
Name	Dilution	Name	Dilution
D-11 (Santa Cruz)	1:200, 2% milk	Anti-Mouse IgG HRP (GE Healthcare)	1:1000, 2% milk
H-70 (Santa Cruz)	1:200, 1% BSA	Anti-Rabbit IgG HRP (GE Healthcare)	1:1000, 2% milk
BXP-21 (Santa Cruz)	1:200, 1% BSA	Anti-Mouse IgG HRP (GE Healthcare)	1:1000, 2% milk
Gβ (Santa Cruz)	1:1000, 2% milk	Anti-Rabbit IgG HRP (GE Healthcare)	1:1000, 2% milk

Table 2: Immunoblotting antibodies and dilutions. BSA: bovine serum albumin

2.6 Functional Transport Assay

Efflux activity of MDR1/P-gp was quantified as previously described by Evseenko, Paxton and Keelan (2006), with slight modifications (Evseenko, Paxton, & Keelan, 2006a). Briefly, 1 million cells/mL of trophoblast cells were cultured in DMEM culture medium supplemented with 10% FBS and 1% antibiotic-antimycotic solution, for 96 hours until complete syncytialization, after which cortisol (500 nM-5μM) was added to the culture medium. After 24 hours of treatment, cells were washed with room temperature HEPES-buffered Tyrode solution, containing Tyrode's salts (Sigma) and 1g $NaHCO_3$ (Sigma). Calcein-AM (Fluka Chemical Corp., Milwaukee, WI, USA) was added to HEPES-buffered Tyrode solution, at a final concentration of 0.4 μM, and cells were incubated for 1 hour, at 37 ^{O}C. When calcein-AM entered into the cell, it was cleaved by esterases in the cytoplasm to form fluorescent calcein, a specific substrate of MDR1/P-gp (**Figure 5**). Cells were washed twice with ice cold HEPES-buffered Tyrode solution and lysed with 10mM Tris•HCl (Sigma)-1% Triton X-100 (Fisher Chemicals) for 15 minutes. Fluorescent calcein accumulation within cells was measured on a fluorescence reader (Spectro Max 2) at 485/535 nm. Cells from individual tissues were cultured in quadruplicate for all treatments. Previous studies in our laboratory have optimized concentration of Calcein-AM and treatment time (Anne Marie Downey, Honours Thesis).

2.7 STATISTICAL ANALYSIS

Data were analyzed and displayed using GraphPad Prism (version 5.04; San Diego, CA, USA). Multiple comparisons were analyzed using one-way ANOVA followed by the Tukey method of post-hoc analyses. Two samples were analyzed using unpaired Student's t-test. Significance was set at $P<0.05$.

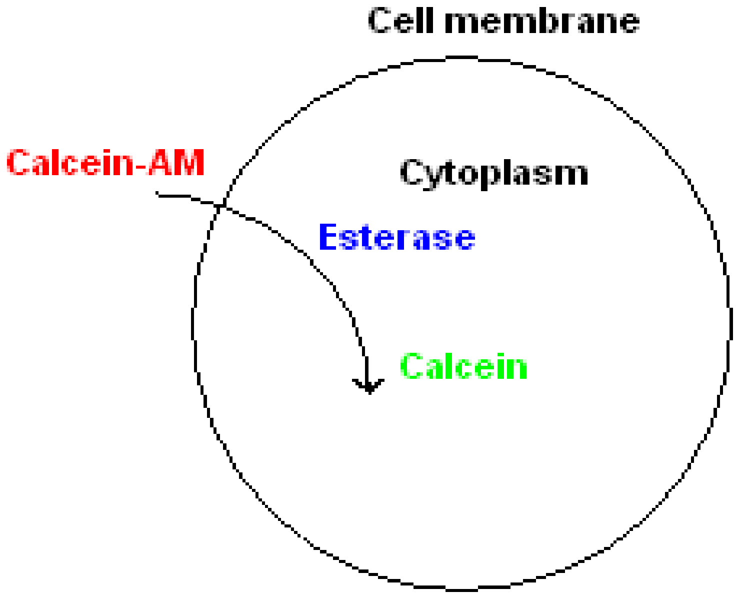

Figure 5| Schematic diagram of conversion of Calcein-AM into fluorescent Calcein by cytoplasmic esterase.

Adopted from Anne Marie Downey (Honours Thesis 2008)

CHAPTER 3: STEROID REGULATION OF MULTIDRUG RESISTANCE PHOSPHOGLYCOPROTEIN (MDR1/P-GP) AND BREAST CANCER RESISTANCE PROTEIN (BCRP) IN THE HUMAN PLACENTA

3.1 RESULTS

3.1.1 General Characterization of Syncytiotrophoblast

Following initial experiments in our laboratory with the G-1 antibody (Santa Cruz), which recognized MDR1/P-gp and MDR3/P-gp, we began using the more specific D-11 antibody, a monoclonal antibody raised against human MDR1/P-gp. To verify the location of MDR1/P-gp in placental tissue, immunohistochemistry was performed using the D-11 antibody. MDR1/P-gp was localized to the syncytiotrophoblast of human placental tissue at both 38 weeks (**Figure 6a**) and 40 weeks of gestation (**Figure 6c**). After 24 hours, trophoblast cells did not completely syncytialize in culture, confirmed by Haemotoxylin staining of the nucleus of individual cells (**Figure 7a**), but they were found to syncytialize after 96 hours in culture (**Figure 7b**). Initial studies were carried out to verify the purity of human trophoblast cells in culture. After 72 hours in culture, IHC was performed using an anti-cytokeratin antibody (**Figure 8b**), which stained trophoblast cells. An anti-vimentin antibody, which detects non-trophoblast cells, did not stain the cells (**Figure 8c**). Syncytialization was confirmed by the presence of multinucleic, anti-desmosomal structures. Desmosomes are cell structures in intracellular junctions. The absence of staining around individual cells and the presence of staining around multiple nuclei indicate syncytialization (**Figure 9b**). MDR1/P-gp was identified in human placental tissue and primary cell culture, by Western blot with the D-11 antibody (**Figure 10**).

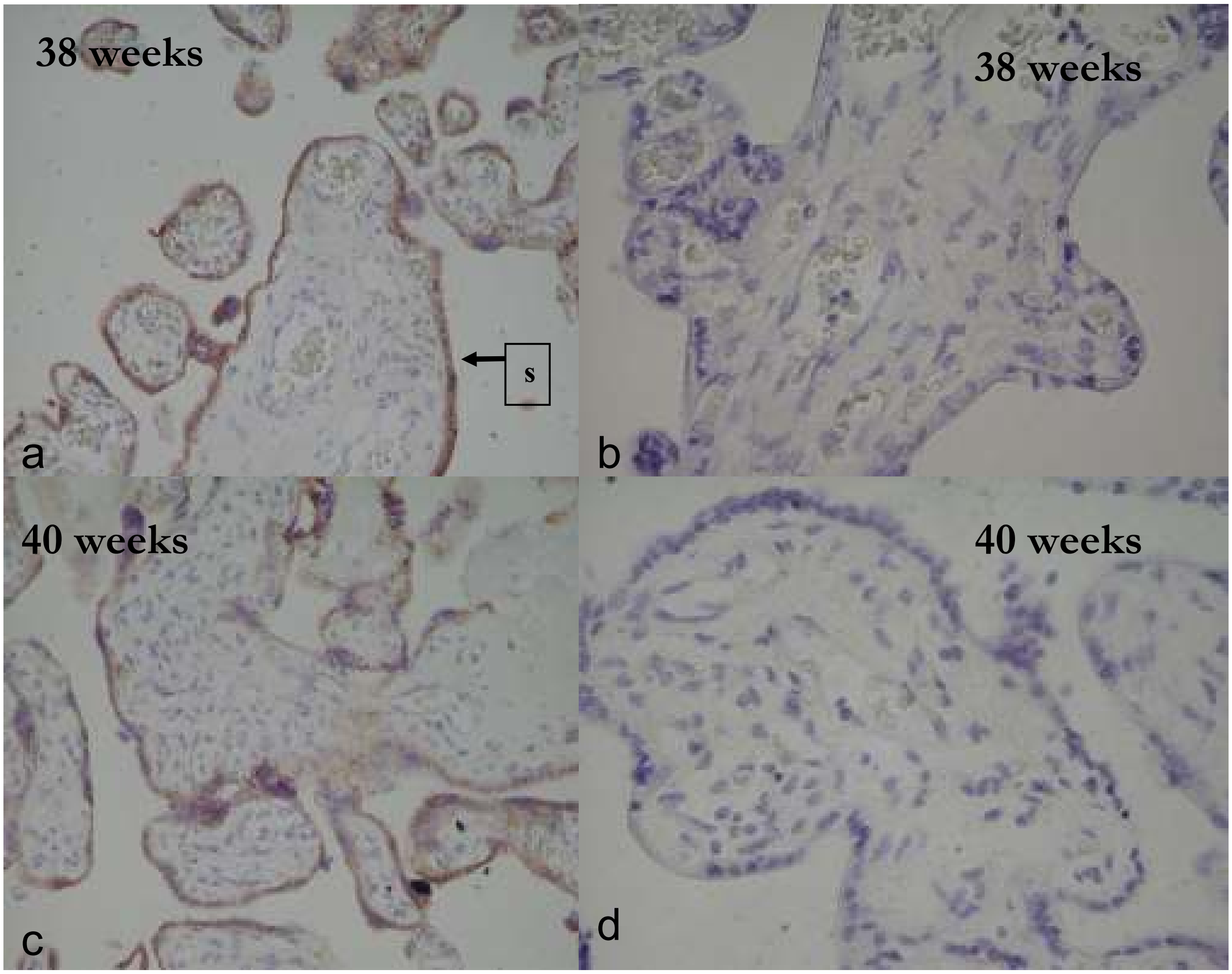

Figure 6| Immunohistochemistry localization of MDR1/P-gp in human placental tissue. Immunohistochemistry localization of MDR1/p-glycoprotein in the human placenta with D-11, a monoclononal anti-MDR1 antibody diluted to 1:500. s:syncytiotrophoblast. A) C. section (38 weeks), b) control C. section (38 weeks, mouse IgG), c) C. section (40 weeks), d) control (mouse IgG).

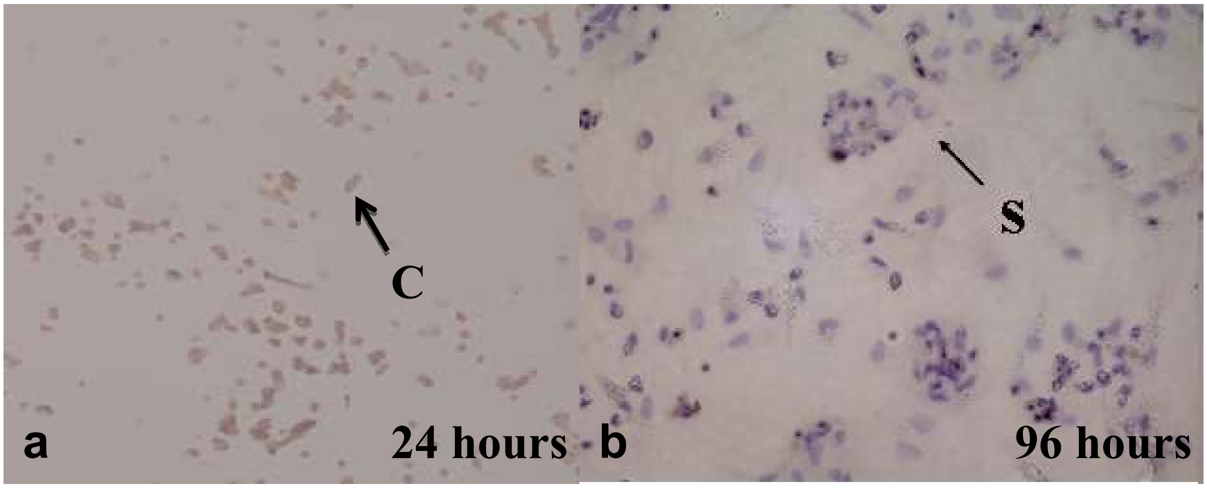

Figure 7| Trophoblast cells in culture. a) Haemotoxylin staining of trophoblast cells after 24 hours in culture. b) staining of trophoblast cells after 96 hours in culture. c: cytotrophoblast, s: syncytiotrophoblast. Figure 6a adopted from Yeboah et al. (2007, unpublished data).

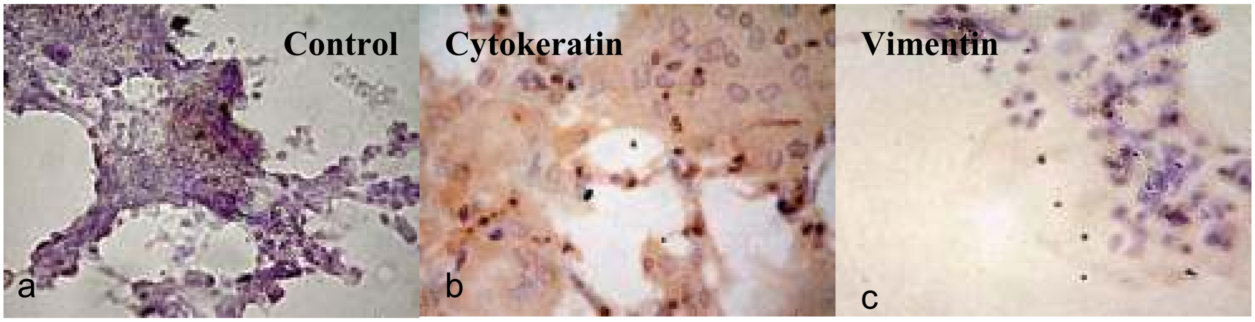

Figure 8| Immunohistochemistry of syncytialized trophoblast primary cell culture. Immunohistochemistry of trophoblast primary cells after 72 hours in culture with a) control (mouse IgG), b) cytokeratin (1:300), and c) vimentin (1:300)

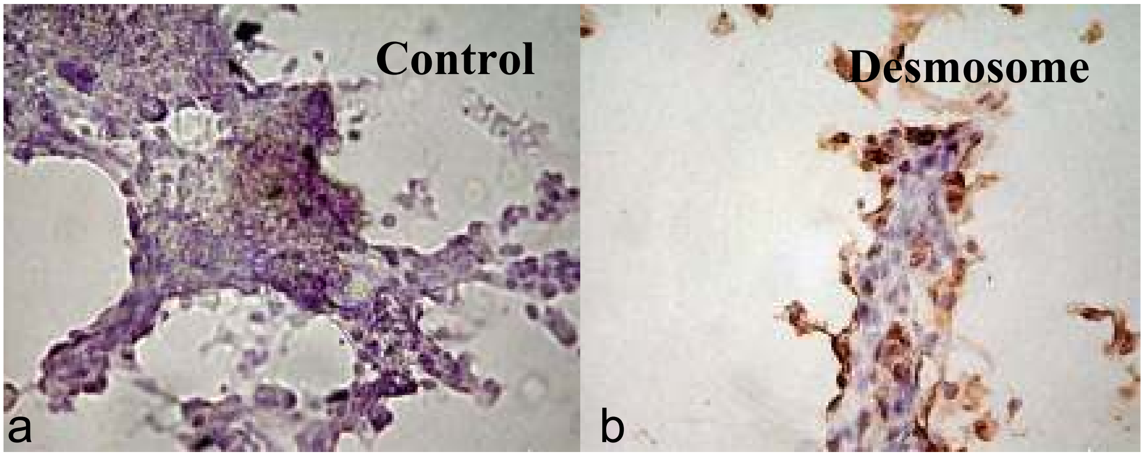

Figure 9| Verification of syncytialized trophoblast cell culture. Immunohistochemistry of trophoblast primary cells after 72 hours in culture with a) control (mouse IgG) and b) anti-desmosomal antibody (1:500)

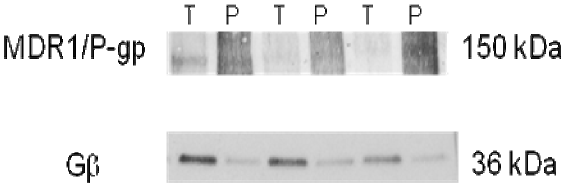

Figure 10| Typical western blot of MDR1/P-gp in human placental tissue and placental primary cell culture. D-11, a monoclonal anti-MDR1/P-gp antibody, was used. 120 μg of tissue protein was loaded and 40 μg of primary cell culture was loaded. Gβ was used as an internal control. T: human placental tissue, P: primary cell culture.

3.1.2 Estrogen and Progesterone Regulation of MDR1/P-gp

Studies from our laboratory have shown that there is a significant decrease in MDR1/P-gp in the human placenta with advancing gestation (Sun *et al.*, 2006); however, how MDR1/P-gp expression is regulated is not known. Estrogen and progesterone levels in maternal serum increase throughout pregnancy (Wang *et al.*, 2006) and glucocorticoids levels also increase in late gestation (Fowden *et al.*, 1998). These steroids have been found to regulate MDR1/P-gp in other cell lines and tissues.

In this study, estrogen and progesterone were investigated as potential regulators of MDR1/P-gp in the human trophoblast cell culture. Western analysis was performed to assess MDR1/P-gp, treated with 0.5 μM, 1 μM, and 5 μM of estradiol for 24 hours, as illustrated in **Figure 11**. Under reducing conditions, the molecular weight of the protein was 150kDa. The level of the 150 kDa protein was quantified by densitometry. Estradiol did not alter MDR1/P-gp levels in human syncytiotrophoblast. Furthermore, Western analysis was also performed to assess MDR1/P-gp in human syncytiotrophoblast, treated with progesterone (500 nM-5 μM) for 24 hours (**Figure 12)**. Progesterone did not have an effect on MDR1/P-gp levels. In combination, estrogen and progesterone were found to regulate MDR1/P-gp in the mouse endometrium (Arceci *et al.*, 1990). To determine the effects of estrogen and progesterone in combination on human syncytiotrophoblast, cells were incubated with a combination of 0.1 μM of both estrogen and progesterone and 5 μM of both estrogen and progesterone for 24 hours. A combination of estrogen and progesterone did not alter MDR1/P-gp levels (**Figure 13**).

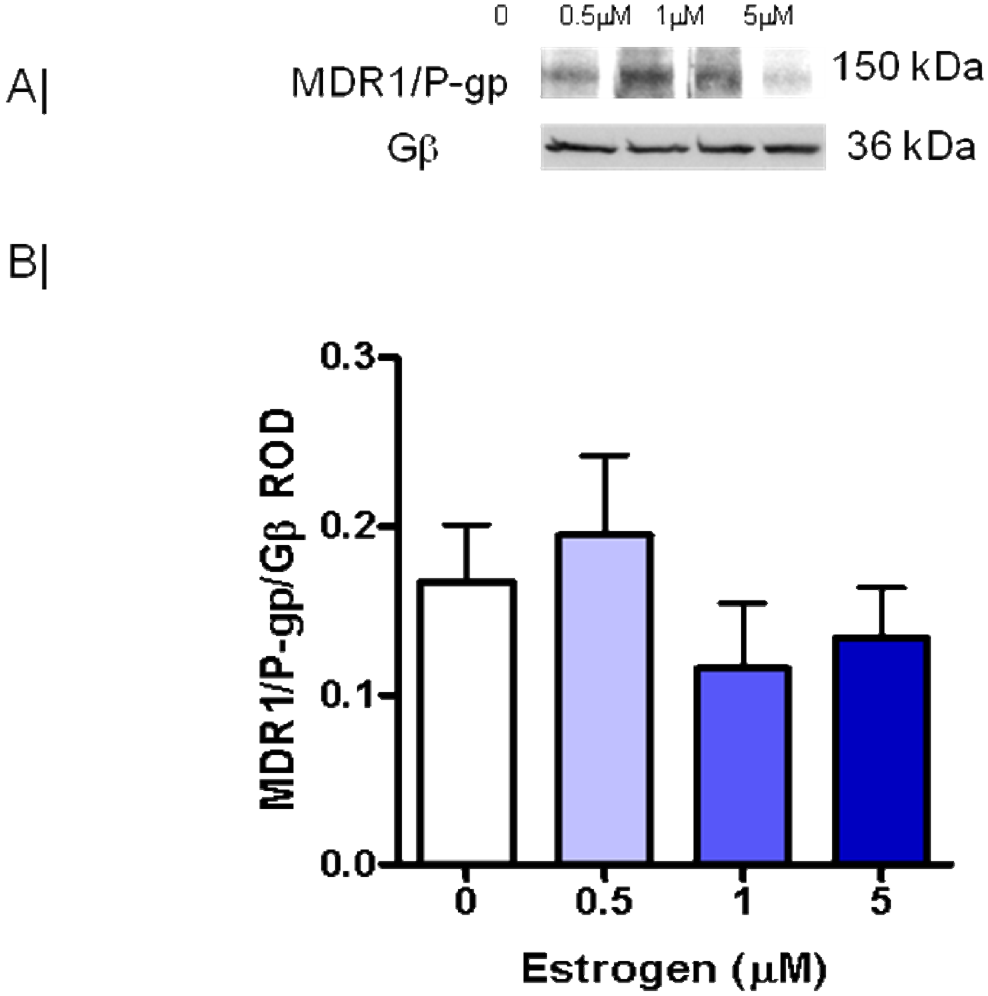

Figure 11| MDR1/P-gp levels in human syncytiotrophoblast after 24 hours of estrogen treatment. A) A typical western blot of MDR1/P-gp and Gβ protein levels after estrogen treatment of 0 (n=4), 0.5 μM (n=4), 1 μM (n=4), and 5 μM (n=4) for 24 hours, using D-11 antibody. B) Quantification of MDR1/P-gp in syncytiotrophoblast. MDR1/P-gp was quantified after 0, 0.5 μM, 1 μM, and 5 μM of estrogen treatment for 24 hours. ANOVA, Bar represents mean +/-SEM, standardized against Gβ and expressed as relative optical density (ROD).

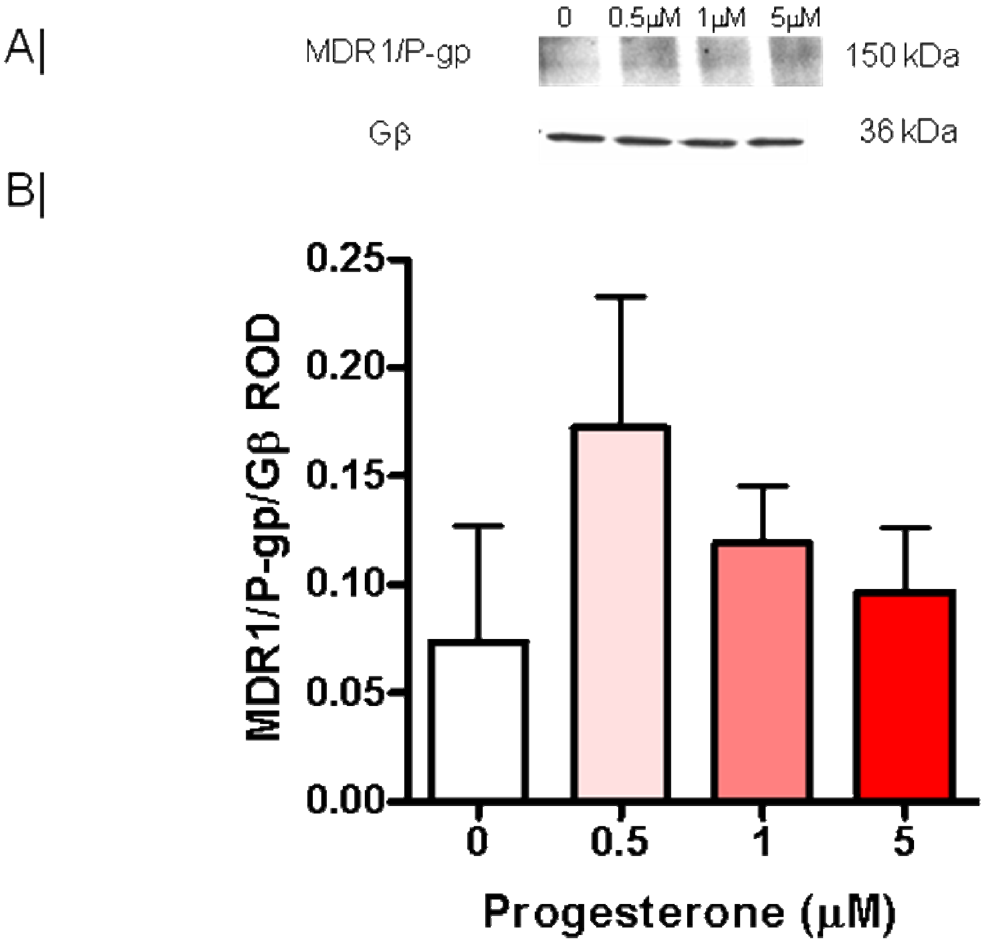

Figure 12| MDR1/P-gp levels in human syncytiotrophoblast after 24 hours of progesterone treatment. A) A typical western blot of MDR1/P-gp and Gβ protein levels after progesterone treatment of 0 (n=4), 0.5 µM (n=4), 1 µM (n=4), and 5 µM (n=4) for 24 hours, using D-11 antibody. B) Quantification of MDR1/P-gp in syncytiotrophoblast. MDR1/P-gp was quantified after 0, 0.5 µM, 1 µM, and 5 µM of progesterone treatment for 24 hours. ANOVA, Bar represents mean +/-SEM, standardized against Gβ and expressed as relative optical density (ROD).

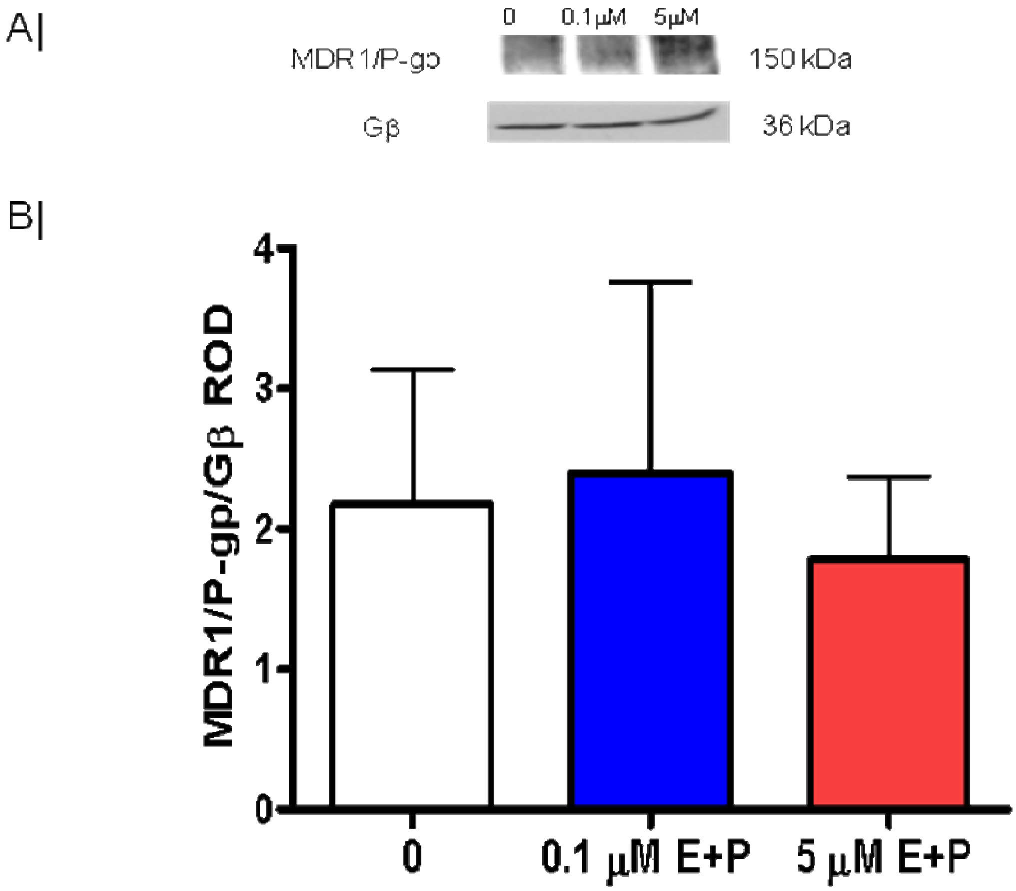

Figure 13| MDR1/P-gp levels in human syncytiotrophoblast after 24 hours of co-incubation with estrogen and progesterone. A) A typical western blot of MDR1/P-gp and Gβ protein levels after a combination of 0 (n=3), 0.1 µM (n=3) of both estrogen and progesterone and 5 µM (n=3) of both estrogen and progesterone for 24 hours, using D-11 antibody. B) Quantification of MDR1/P-gp in syncytiotrophoblast. MDR1/P-gp was quantified after 0, 0.1 µM, and 5 µM of co-incubation of estrogen and progesterone for 24 hours. ANOVA, Bar represents mean +/-SEM, standardized against Gβ and expressed as relative optical density (ROD). E: Estrogen, P: Progesterone.

3.1.3 Glucocorticoid Regulation of MDR1/P-gp

Cortisol levels increase at the end of pregnancy (Fowden *et al.*, 1998) and glucocorticoids have been implicated in MDR1/P-gp regulation other tissues and *in vivo*. To investigate the effect of cortisol on MDR1/P-gp in human syncytiotrophoblast, cells were treated with 0.5 μM, 1 μM, and 5 μM of cortisol for 24 hours. A high level of cortisol (5 μM) significantly decreased MDR1/P-gp levels ($p<0.05$), compared to the control (**Figure 14)**. Other groups have suggested that cortisol may affect the syncytialization of trophoblast cells in culture (J. Challis, personal communication), which could potentially alter MDR1/P-gp. To examine if cortisol altered syncytialization of trophoblast cells, we incubated cells with a high concentration of cortisol (10 μM) from 24-96 hours of culture. After 96 hours in culture, syncytialization of trophoblast cells was not affected by 10 μM of cortisol, as verified by Haemotoxylin staining (**Figure 15)**.

The mechanism by which cortisol regulates MDR1/P-gp in the placenta was investigated. Cells were incubated with dexamethasone, a synthetic glucocorticoid and a known agonist of the glucocorticoid receptor (GR) receptor (Frego & Davidson, 2006), to determine if the effects of dexamethasone paralleled the effects of cortisol on MDR1/P-gp regulation under the same conditions that cortisol caused a decrease in MDR1/P-gp levels. Dexamethasone (0.5-5 μM) did not affect MDR1/P-gp (**Figure 16**). Furthermore, cells were co-incubated with cortisol and RU486, a competitive antagonist of the GR receptor (Beck *et al.*, 1993). Co-incubation of cortisol (5 μM) and RU486 (5 μM) did not reverse the inhibitory effect of cortisol ($p<0.01$), as illustrated in **Figure 17**.

Another potential mechanism by which cortisol could regulate MDR1/P-gp in human syncytiotrophoblast is via the mineralocorticoid receptor (MR). Cell were co-incubated with spironolactone, a mineralocorticoid receptor antagonist (Delyani, 2000), and cortisol, to determine if spironolactone would alter cortisol's regulation of MDR1/P-gp. Western analysis was performed to assess MDR1/P-gp in human syncytiotrophoblast treated with 5 μM of cortisol for 24 hours and a combination of 5 μM of spironolactone and 5 μM of cortisol for 24 hours (**Figure 18**). In this study the results were variable and MDR1/P-gp cortisol alone, nor the combination of cortisol and spironolactone, significantly altered MDR1/P-gp levels. However, in all instances the levels of MDR1/P-gp in the treated cells appeared lower.

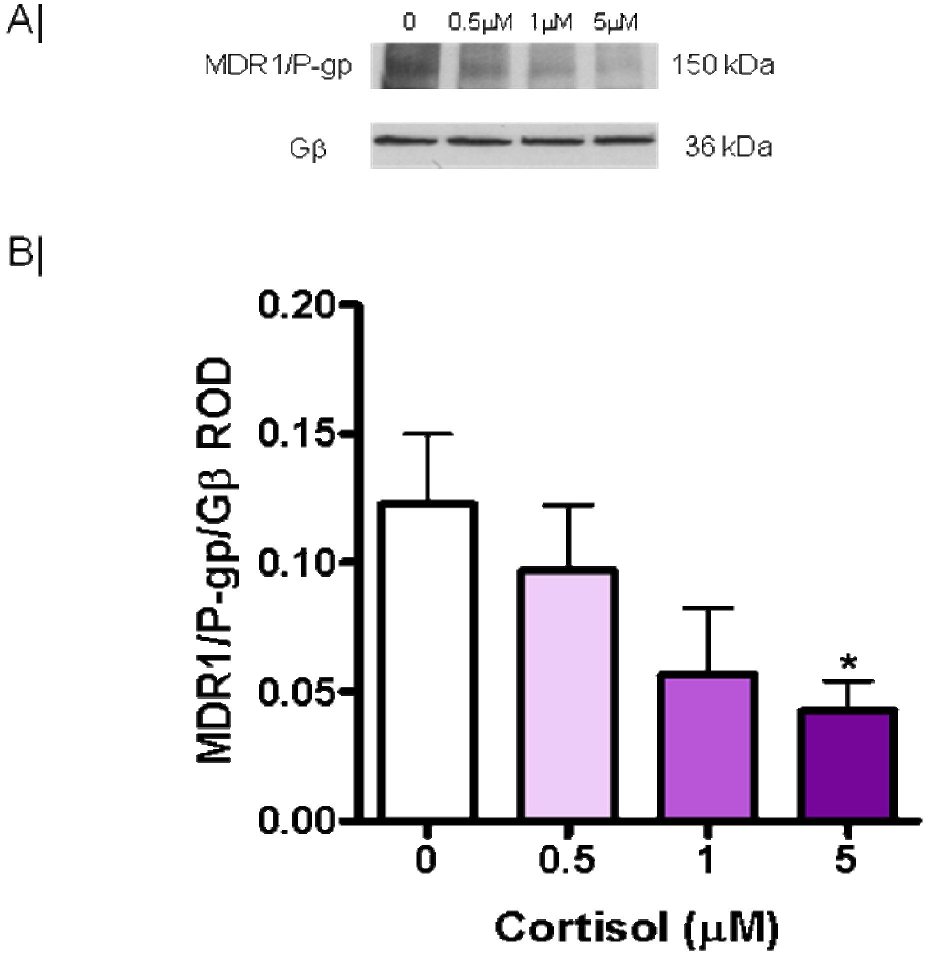

Figure 14| MDR1/P-gp levels in human syncytiotrophoblast after 24 hours of cortisol treatment. A) A typical western blot of MDR1/P-gp and Gβ protein levels after cortisol treatment of 0 (n=4), 0.5 µM (n=4), 1 µM (n=4), and 5 µM (n=4) for 24 hours, using D-11 antibody. B) Quantification of MDR1/P-gp in syncytiotrophoblast. MDR1/P-gp was quantified after 0, 0.5 µM, 1 µM, and 5 µM of cortisol treatment for 24 hours. ANOVA, Bar represents mean +/-SEM, standardized against Gβ and expressed as relative optical density (ROD).*=$p<0.05$.

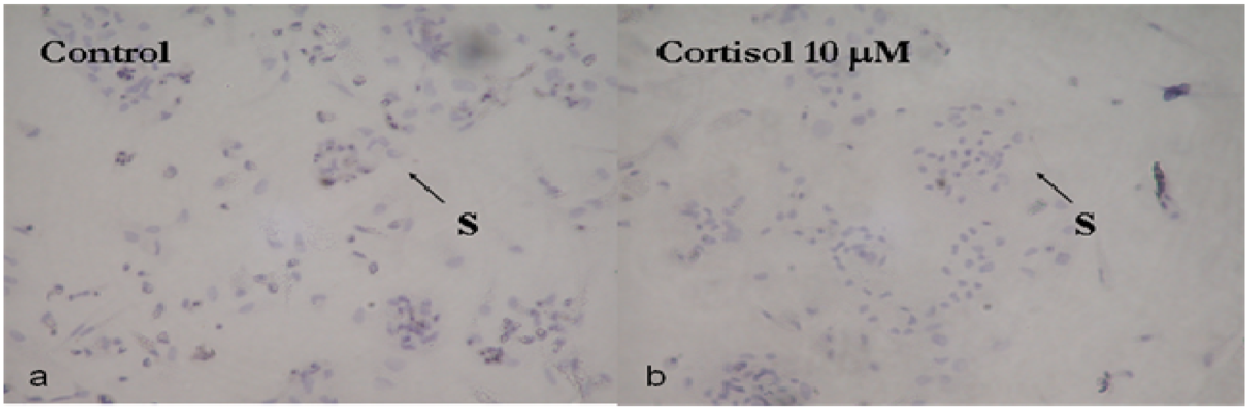

Figure 15| Syncytialization of trophoblast primary cells after 96 hours in culture. s: syncytiotrophoblast. Immunostaining of syncytiotrophoblasts in culture a) with mouse IgG b) after treatment with 10 μM of cortisol,with mouse IgG.

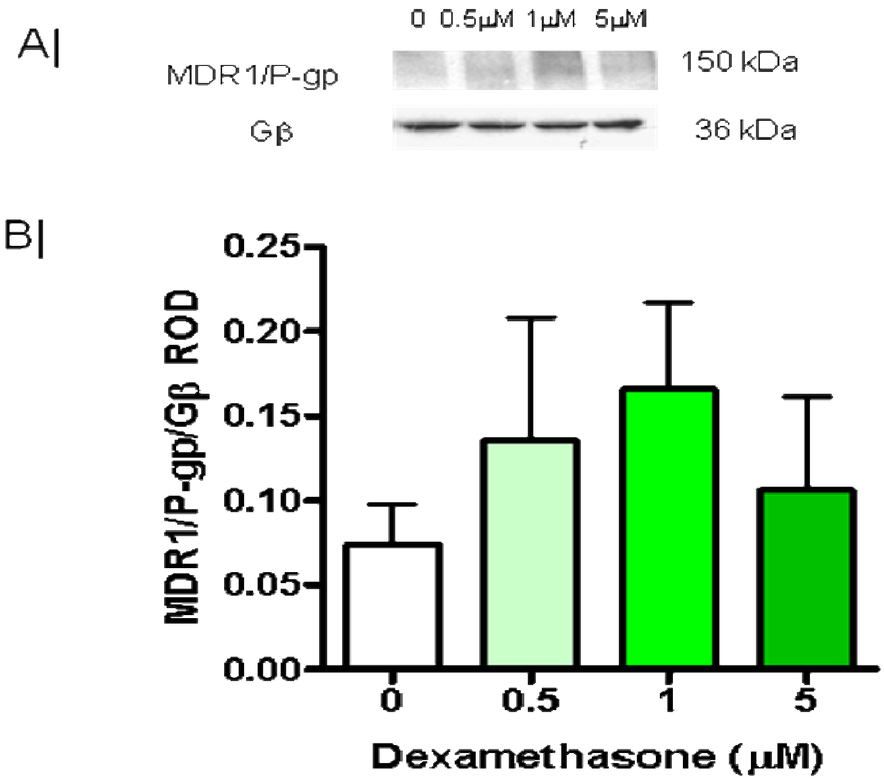

Figure 16| MDR1/P-gp levels in human syncytiotrophoblast after 24 hours of dexamethasone treatment. Quantification of MDR1/P-gp in syncytiotrophoblast. MDR1/P-gp was quantified after 0 (n=8), 0.5 μM (n=8), 1 μM (n=8), and 5 μM (n=8) of dexamethasone treatment for 24 hours. ANOVA, Bar represents mean +/-SEM, standardized against Gβ and expressed as relative optical density (ROD).

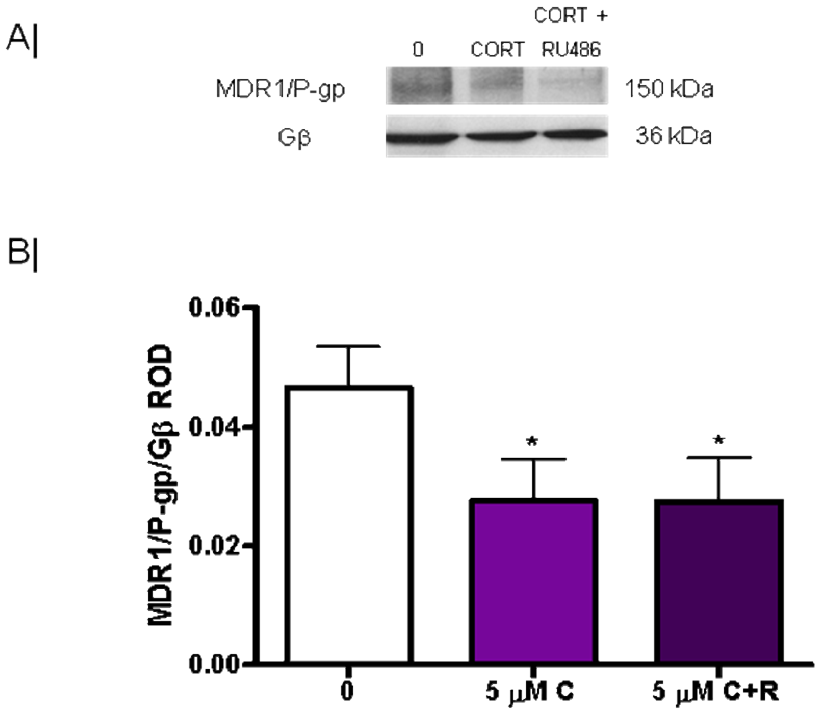

Figure 17| MDR1/P-gp levels in human syncytiotrophoblast after 24 hours of cortisol and cortisol + RU486 treatment. A) A typical western blot of MDR1/P-gp and Gβ protein levels after cortisol treatment 5 μM (n=4) and a combination of cortisol, 5 μM (n=4), and RU486, 5 μM (n=4), for 24 hours, using D-11 antibody. **B)** Quantification of MDR1/P-gp in syncytiotrophoblast. MDR1/P-gp was quantified after 5 μM of cortisol treatment and 5 μM of both cortisol and RU486 treatment for 24 hours. C: cortisol, R:RU486. ANOVA, Bar represents mean +/-SEM, standardized against Gβ and expressed as relative optical density (ROD). *=$p<0.01$.

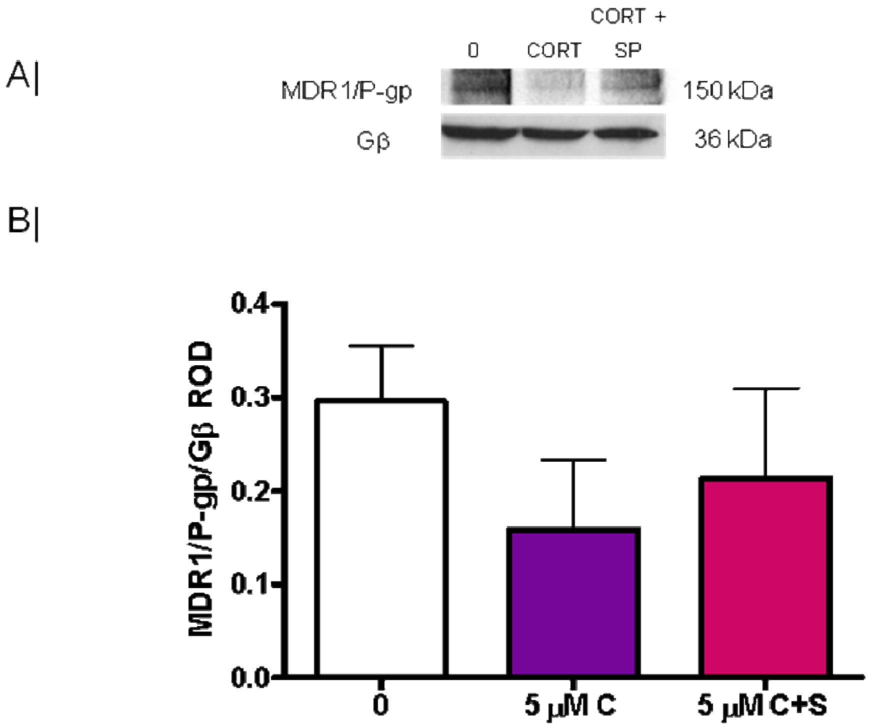

Figure 18| MDR1/P-gp levels in human syncytiotrophoblast after 24 hours of cortisol and cortisol + spironolactone treatment. **A)** A typical western blot of MDR1/P-gp and Gβ protein levels after cortisol treatment 5 µM (n=4) and a combination of cortisol, 5 µM (n=4), and spironolactone, 5 µM (n=4), for 24 hours, using D-11 antibody. **B)** Quantification of MDR1/P-gp in syncytiotrophoblast. MDR1/P-gp was quantified after 5 µM of cortisol treatment and 5 µM of both cortisol and spironolactone treatment for 24 hours. C:cortisol, SP:spironolactone. ANOVA, Bar represents mean +/-SEM, standardized against Gβ and expressed as relative optical density (ROD).

3.1.4 Function of MDR1/P-gp

In order to determine if the decrease in MDR1/P-gp levels altered function, transport studies were then used to determine the functional activity of MDR1/P-gp in culture. Human syncytiotrophoblast were incubated with calcein-AM for 1 hour, after 24 hours pretreatment with cortisol. When calcein-AM enters into the cell, it is cleaved by esterases to form fluorescent calcein (**Figure 5**) (Evseenko, Paxton, & Keelan, 2006b). Calcein is a substrate for MDR1/P-gp. If MDR1/P-gp is upregulated, calcein is effluxed from the cells, whereas if MDR1/P-gp is downregulated calcein will accumulate inside the cells. Fluorescence accumulation is represented as a ratio of treated to control. After pretreatment with 0.5 μM, 1 μM, and 5 μM of cortisol, there was not a statistically significant effect of cortisol on fluorescence accumulation (**Figure 19**).

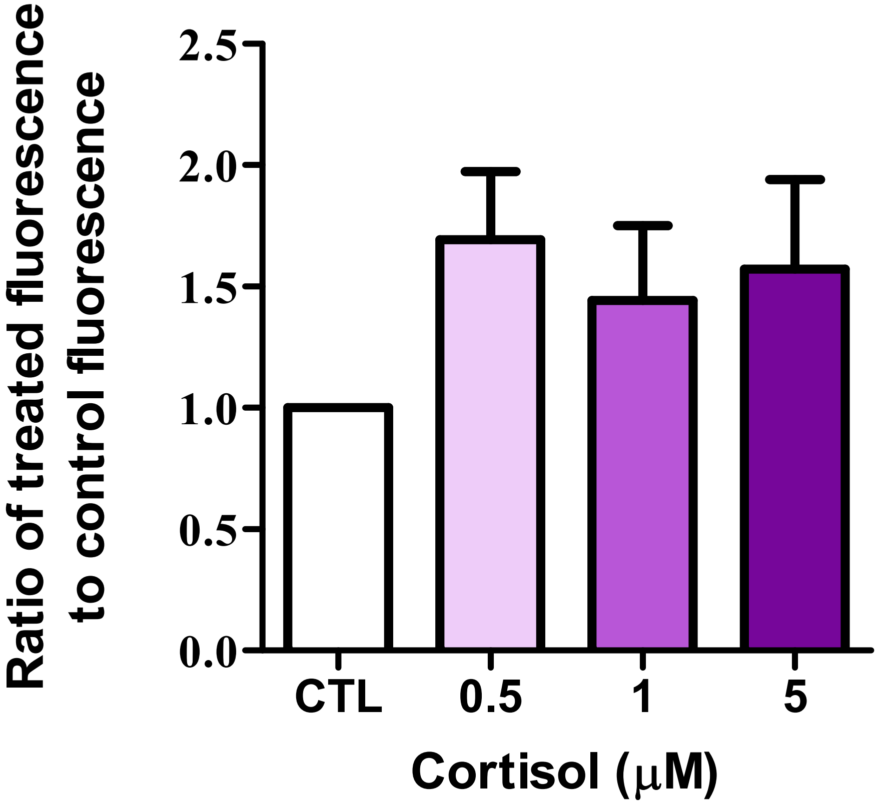

Figure 19| Fluorescence accumulation of calcein in syncytiotrophoblast treated with cortisol for 24 hours. Fluorescence accumulation of calcein in syncytiotrophoblast treated with 0 (n=7), 0.5 μM (n=7), 1 μM (n=7), and 5 μM (n=7) of cortisol for 24 hours. Cultures incubated with calcein-AM for 1 hour. Each n value is a mean of quadruplicate samples from one placental culture. Data is shown as a ratio of treated to control. ANOVA, Mean+/-SEM

3.1.5 Estrogen and Progesterone Regulation of BCRP

BCRP was found in primary trophoblast cell culture and placental tissue, using the H-70 antibody (**Figure 20**). Studies from our laboratory have shown that there is a significant increase in BCRP in the human placenta with advancing gestation (Yeboah *et al.*, 2006a). The physiological regulator of BCRP is not known. Estrogen, progesterone, and glucocorticoids increase throughout pregnancy (H. Wang *et al.*, 2006; Fowden *et al.*, 1998), and may potentially regulate BCRP in the human placenta. To investigate if estrogen and progesterone regulates BCRP in human syncytiotrophoblast, cells were incubated with 0.5 μM, 1 μM, and 5 μM of estrogen for 24 hours (**Figure 21**). Under reducing condition, the molecular weight of the protein was 72 kDa. The level of the 72 kDa protein was quantified by densitometry. Estrogen did not have a significant effect on BCRP levels in human syncytiotrophoblast after 24 hours. Western analysis was performed after cells were treated with 0.5 μM, 1 μM, and 5 μM of progesterone for 24 hours, as illustrated in **Figure 22**. Progesterone did not have a significant effect on BCRP levels in human syncytiotrophoblast after 24 hours. Occasionally, a double band was observed, with one band at approximately 72 kDa and one between 75-80 kDa.

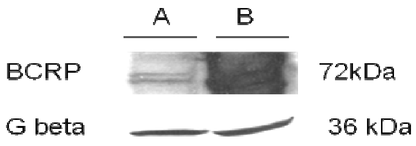

Figure 20| Typical western blot of BCRP in placental primary cell culture and placental tissue. H-70, a polyclonal anti-BCRP antibody, was used. 20 μg of protein was loaded in each lane. Gβ was used as an internal control. A: Primary cell culture, B: Placental Tissue.

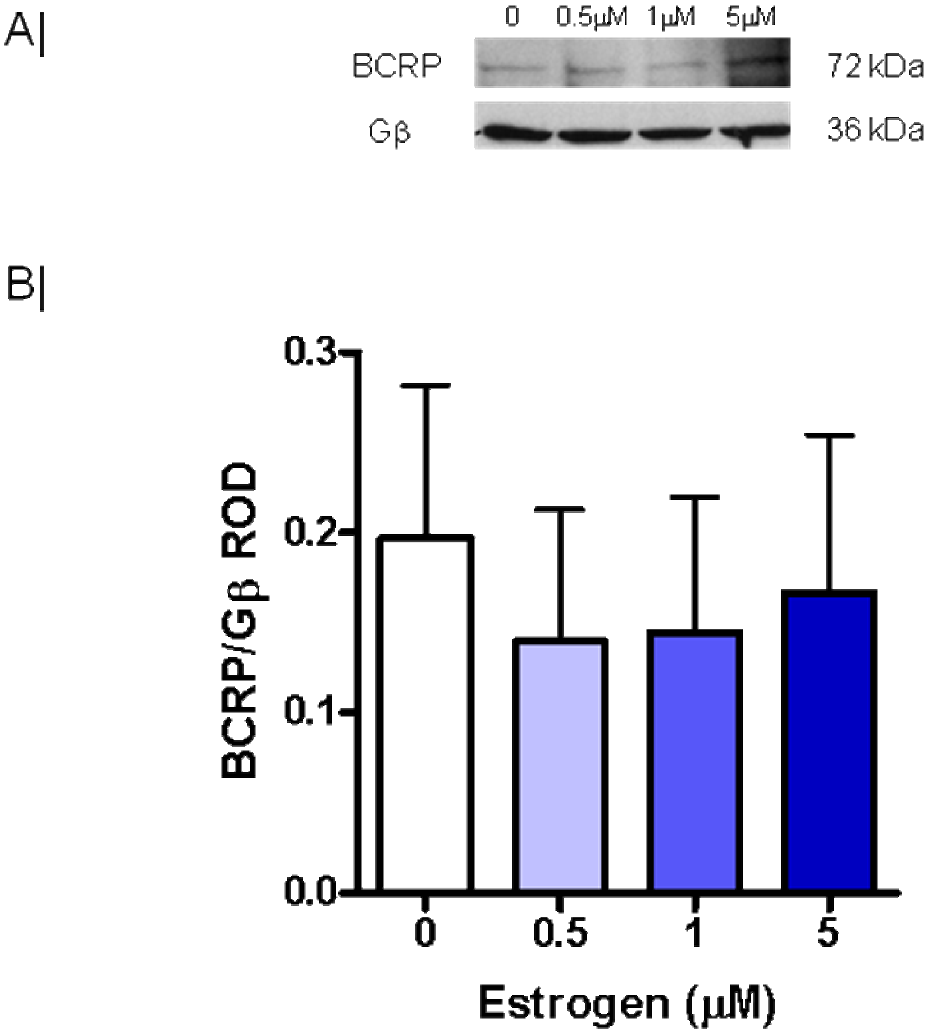

Figure 21| BCRP levels in human syncytiotrophoblast after 24 hours of estrogen treatment. **A)** A typical western blot of BCRP and Gβ protein levels after estrogen treatment of 0 (n=4), 0.5 μM (n=4), 1 μM (n=4), and 5 μM (n=4) for 24 hours, using H-70 antibody. **B)** Quantification of BCRP in syncytiotrophoblast. BCRP was quantified after 0, 0.5 μM, 1 μM, and 5 μM of estrogen treatment for 24 hours. ANOVA, Bar represents mean +/- SEM, standardized against Gβ and expressed as relative optical density (ROD).

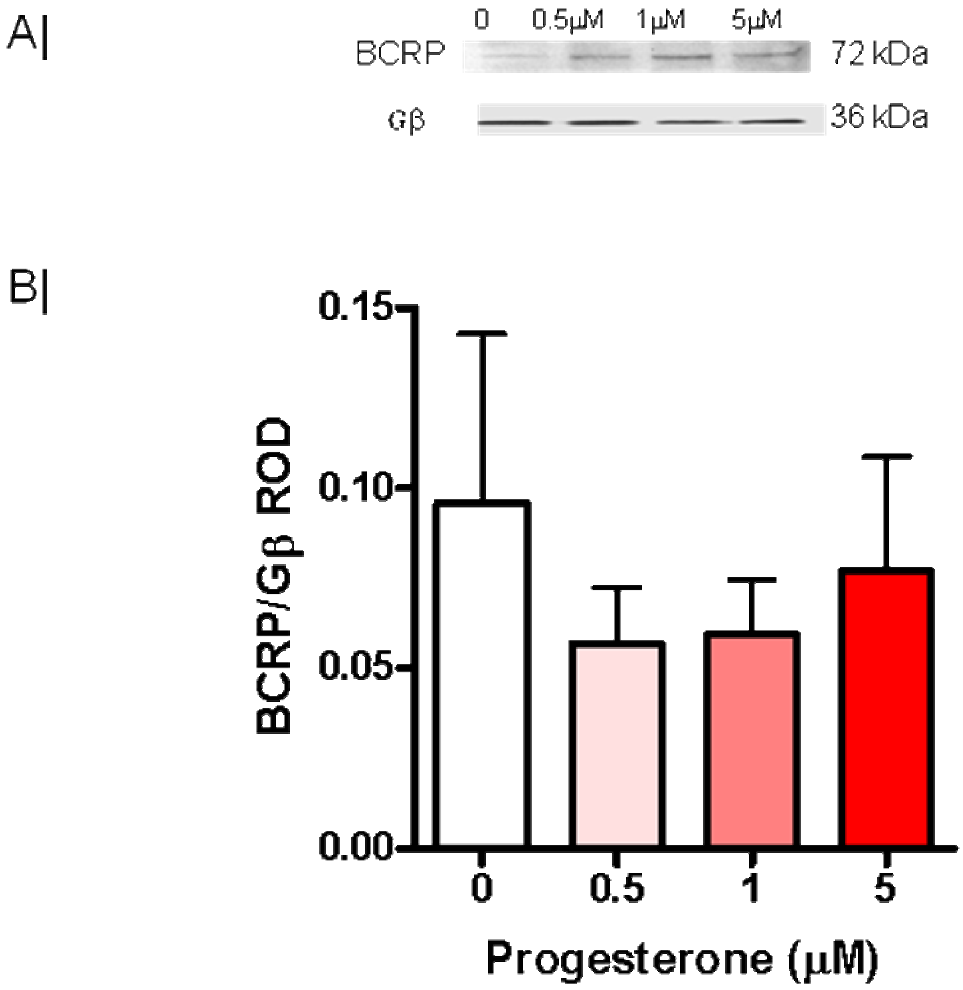

Figure 22| BCRP levels in human syncytiotrophoblast after 24 hours of progesterone treatment. A) A typical western blot of BCRP and Gβ protein levels after progesterone treatment of 0 (n=4), 0.5 μM (n=4), 1 μM (n=4), and 5 μM (n=4) for 24 hours, using H-70 antibody. **B)** Quantification of BCRP in syncytiotrophoblast. BCRP was quantified after 0, 0.5 μM, 1 μM, and 5 μM of progesterone treatment for 24 hours. ANOVA, Bar represents mean +/-SEM, standardized against Gβ and expressed as relative optical density (ROD).

3.1.6 Glucocorticoid Regulation of BCRP

To investigate glucocorticoid regulation of BCRP, western analysis was performed after human syncytiotrophoblast cells were incubated with cortisol or dexamethasone. Cortisol (0.5-5 μM) did not have a significant effect on BCRP levels in human syncytiotrophoblast after 24 hours (**Figure 23).** Similarly, dexamethasone did not have a significant effect on BCRP levels, as shown in **Figure 24**. A double band was inconsistently observed, with one band at approximately 72 kDa and one between 75-80 kDa.

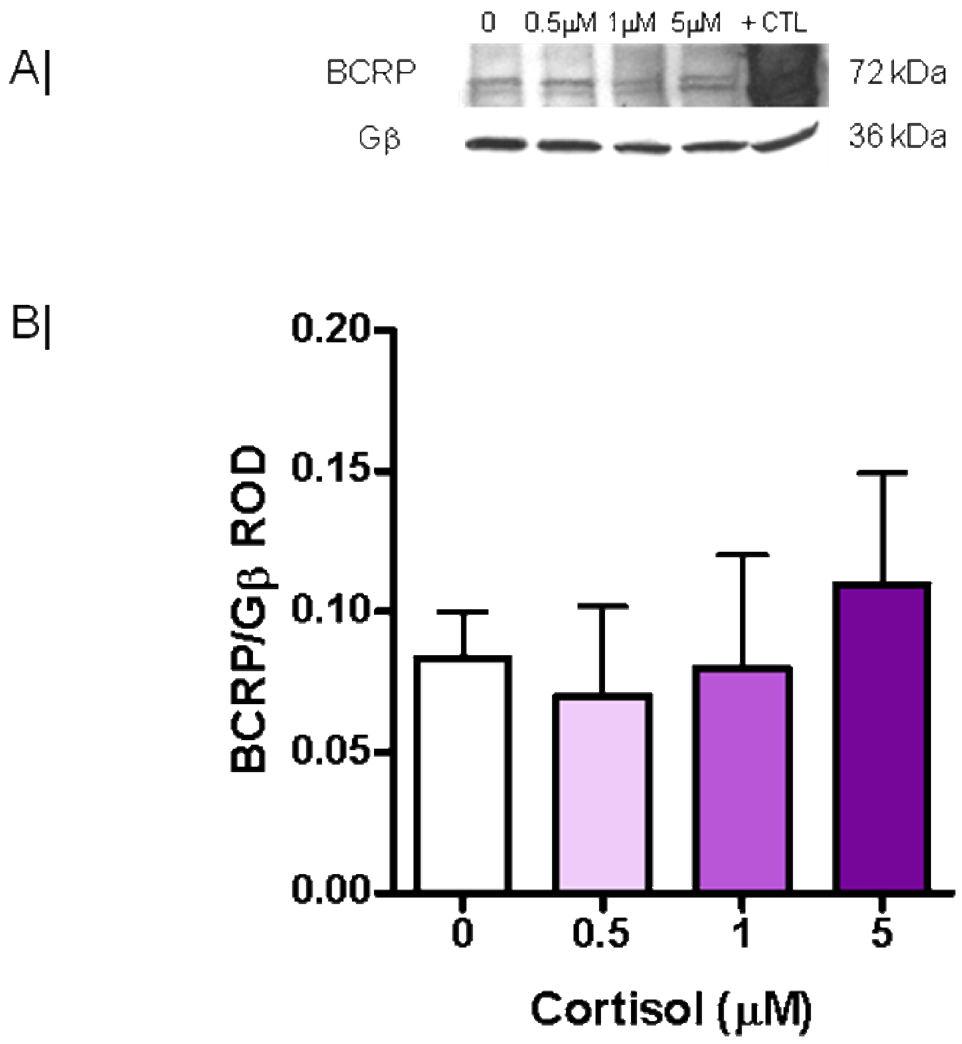

Figure 23| BCRP levels in human syncytiotrophoblast after 24 hours of cortisol treatment. **A)** A typical western blot of BCRP and Gβ protein levels after cortisol treatment of 0 (n=4), 0.5 μM (n=4), 1 μM (n=4), and 5 μM (n=4) for 24 hours, using H-70 antibody. Placental tissue was used as a positive control. **B)** Quantification of BCRP in syncytiotrophoblast. BCRP was quantified after 0, 0.5 μM, 1 μM, and 5 μM of cortisol treatment for 24 hours. ANOVA, Bar represents mean +/-SEM, standardized against Gβ and expressed as relative optical density (ROD).

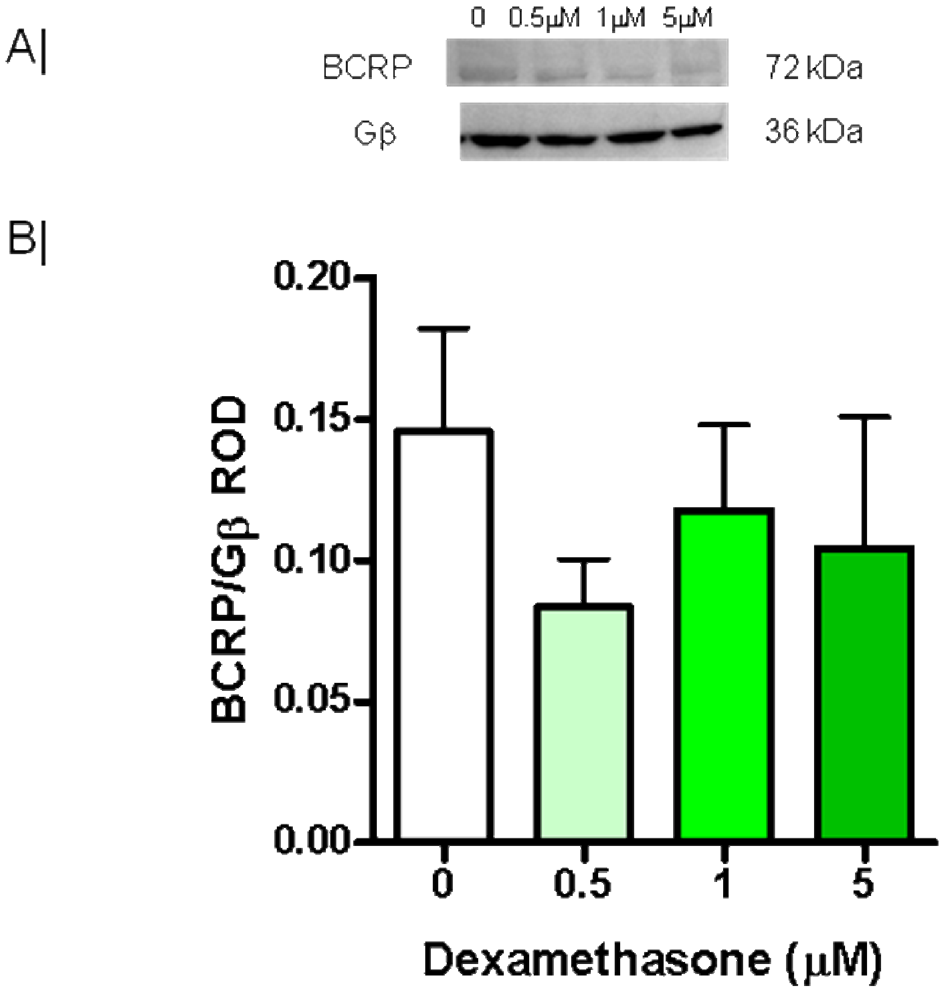

Figure 24| BCRP levels in human syncytiotrophoblast after 24 hours of dexmathesone treatment. A) A typical western blot of BCRP and Gβ protein levels after dexamethasone treatment of 0 (n=4), 0.5 μM (n=4), 1 μM (n=4), and 5 μM (n=4) for 24 hours, using H-70 antibody. **B)** Quantification of BCRP in syncytiotrophoblast. BCRP was quantified after 0, 0.5 μM, 1 μM, and 5 μM of dexamethasone treatment for 24 hours. ANOVA, Bar represents mean +/-SEM, standardized against Gβ and expressed as relative optical density (ROD).

3.2 DISCUSSION

There is limited information regarding the physiological regulation of ABC transporters, MDR1/P-gp and BCRP, in the human placenta. Therefore, our main objectives were to determine if maternal steroids, which are known to increase throughout pregnancy (Wang *et al.*, 2006; Fowden *et al.*, 1998), regulate MDR1/P-gp and/or BCRP in the human placenta. Specifically, the regulatory effects of progesterone, estrogen, and glucocorticoids were studied in human syncytiotrophoblast cultures. Our results indicate that estrogen and progesterone did not alter MDR1/P-gp levels in human syncytiotrophoblast, but cortisol decreased MDR1/P-gp levels. It did not appear that cortisol acted via the GR or MR to exert its regulatory effect. Furthermore, MDR1/P-gp function decreased, but this decrease was not statisically significant. We also demonstrated that maternal steroids did not have a regulatory effect on BCRP levels. Human studies relied on *in vitro* cultures, using a well defined system.

3.2.1 Syncytialization and purity of trophoblast cells in culture

Kliman *et al.* (1986) found that trophoblast cells in culture form syncytium at 24-48 hours (Kliman *et al.*, 1986). Other groups have used 72 hours for syncytialization of trophoblast cells in culture, before extracting protein for western blot analysis (Evseenko *et al.*, 2007) . We wanted to establish a timeframe in our laboratory, in which we could ensure that trophoblast cells were completely syncytialized in culture, and this timeframe would be consistently used throughout the study. Our results indicate, by staining cells with Haemotoxylin and IHC using an anti-desmosomal cytokeratin antibody, that 72-96 hours was a sufficient amount of time for syncytialization of trophoblast cells *in vitro*. Therefore, steroid treatment began at 96 hours, after syncytialization. Evseenko, Paxton and Keelan (2007) began treating trophoblast cells in culture, with estrogen and progesterone, after 24 hours (Evseenko *et al.*, 2007). Results from our laboratory have indicated that trophoblast cells are not completely syncytialized at 24 hours, as individual cells are still visible using Haemotoxylin staining. This could indicate that trophoblast cells, at 24 hours *in vitro*, were not a true representation of human syncytiotrophoblast.

The Kliman method of isolating trophoblast cells from human placental tissue has been used successfully by other groups (Kliman *et al.*, 1986; Premyslova *et al.*, 2003) (Evseenko *et al.*, 2007). Our results suggest that the Kliman method, with modifications, is successful in isolating a pure culture of trophoblast cells. Cytokeratin is an intermediate filament found in the cytoskeleton of epithelial cells and present in syncytiotrophoblast (Khong, Lane, & Robertson, 1986). Our culture stained positively for cytokeratin after 72 hours and there was an absence of vimentin staining, which indicated that we had highly purified cultures.

3.2.2 MDR1/P-gp in Human Placental Tissue and Trophoblast Primary Cell Culture

Previous studies in our laboratory localized MDR1/P-gp to syncytiotrophoblast in human placental tissue (Sun *et al.*, 2006), using an antibody that detected both MDR1/P-gp and MDR3/P-gp, an ABC transporter located on the basolateral membrane of human syncytiotrophoblast (Evseenko, Paxton, & Keelan, 2006a). Our results also detected MDR1/P-gp in syncytiotrophoblast of human placental tissue, using a more specific D-11 antibody, a monoclonal antibody raised against human MDR1/P-gp. Furthermore, MDR1/P-gp was detected in human placental tissue and trophoblast cells syncytialized for 96 hours, using Western blot and the same antibody. Throughout this study, MDR1/P-gp was consistently detected at 150 kDa, by Western blot, and some studies have suggested that MDR1/P-gp can be found between 140-170 kDa (Ling *et al.*, 1983). MDR1/P-gp is also N-glycosylated at the first extracellular loop (Schinkel & Jonker, 2003) and this may account for the differences in molecular weight found between these studies.

3.2.3 Steroid Regulation of MDR1/P-gp

There is limited information regarding estrogen and progesterone regulation of MDR1/P-gp in humans. Estrogen and progesterone increase towards the end of pregnancy, in maternal circulation (Wang *et al.*, 2006), and could possibly be implicated in the decrease of MDR1/P-gp levels described towards term in the human placenta (Sun *et al.*, 2006). There are a few studies that implicate estrogen in the regulation of MDR1/P-gp in cell lines (Biing *et al.*, 1994; Kim & Benet, 2004; Zampieri *et al.*, 2002; Mutoh *et al.*, 2006), however these results are contradictory. For example, Kim *et al.* (2004) found that 50 μM of ethynyl estradiol induced MDR1/P-gp levels in the LS180, a human colon cancer cell line (Kim & Benet, 2004). However, in another study, MDR1/P-gp levels were down-regulated in two MDR1/P-gp induced, estrogen receptor α positive human breast cancer cell lines, MCF-7/MDR and T-47D/MDR, by 100 pM of estradiol (Mutoh *et al.*, 2006). These divergences between studies could be attributed to the differences in concentrations of estradiol or cell lines used, yet there are no consistent findings related to estrogen regulation of MDR1/P-gp. In regards to the human placenta, one study has shown that estrogen (100 nM) upregulated MDR1/P-gp in human trophoblast cell culture, after 48 hours treatment (Evseenko *et al.*, 2007). Our results do not support these findings; we observed that estrogen (500 nM-5μM) did not have a regulatory effect on MDR1/P-gp levels, in human syncytiotrophoblast. The differences in the results could be contributed to the fact that Evseenko, Paxton and Keelan (2007) treated trophoblast cells after 24 hours in culture, which we have found to be an insufficient amount of time for syncytialization to occur and may not have been a true representation of syncytiotrophoblast *in vitro*. The concentration of estrogen used by Evseenko, Paxton and Keelan (2007) was based on the concentration of estrogen in maternal serum during pregnancy; however, we used a higher concentration of estrogen based on the fact that the concentration of steroids in steroid-producing tissue is probably much higher than in maternal serum (Yang *et al.*, 1989).

As opposed to humans, where maternally circulating progesterone levels increase towards term, progesterone levels in the mouse decrease significantly from mid-gestation to term. This decrease in progesterone concentration in maternal serum positively correlated with the decrease in *Mdr1b* mRNA (the main isoform in the mouse placenta), which implies a potential progesterone regulation of *Mdr1b* mRNA and Mdr1b (Kalabis *et al.*, 2005). However, *in vivo,* progesterone was found to have no effect on *Mdr1* mRNA expression and Mdr1/P-gp levels in the mouse placenta (Petropoulos *et al.*, 2007). These results were confirmed in the rat placenta, where *Mdr1a* and *Mdr1b* mRNA expression was not affected by partial progesterone withdrawal (Mark *et al.*, 2009). Similarly, our results indicate that progesterone does not regulate MDR1/P-gp in human syncytiotrophoblast. Progesterone has been shown to regulate MDR1/P-gp in transfected T47D, a human breast cancer cell line, and HeLa, human cervical cell line (Mallick & Horwitz, 1997; Piekarz *et al.*, 1993) and in the uterine secretory epithelium of the mouse (Yang *et al.*, 1989). The differences in these findings and our results may be due to cell or tissue-specific regulation of MDR1/P-gp. A concentration of 10 nM-1 μM was shown to up-regulate MDR1/P-gp in granulosa cells from porcine ovarian follicles (Fukuda *et al.*, 2006), while a progesterone concentration of 5-50 μM down-regulates MDR1/P-gp in a murine macrophage-like cell line (Yang *et al.*, 1989) , *in vitro.* Progesterone was found to upregulate MDR1/P-gp levels in human trophoblast culture (Evseenko *et al.*, 2007), however, as previously stated, they treated cultured trophoblast cells after 24 hours in culture, which may account for the disparity between findings. Furthermore, the differences in concentration of progesterone used in the two studies could be a source of discrepancy. Evseenko, Paxton and Keelan (2007) used a concentration of 100 nM of progesterone, whereas 500 nM-5μM of progesterone was used in this study. This range of progesterone concentrations was decided upon based past *in vitro* studies.

Estrogen and progesterone, in combination, have been found to upregulate Mdr1/P-gp in the mouse endometrium (Arceci *et al.*, 1990). In the present study, co-incubation of human syncytiotrophoblast with both estrogen and progesterone did not regulate MDR1/P-gp levels.

Endogenous cortisol levels increase exponentially in late pregnancy (Fowden *et al.*, 1998) and are believed to be involved in fetal lung maturation and the onset of labour. The increase in cortisol coincides with the down-regulation in MDR1/P-gp levels in the human placenta, previously described by our laboratory (Sun *et al.*, 2006), indicating a potential role of cortisol in the physiological regulation of MDR1/P-gp. The present study found that cortisol downregulated MDR1/P-gp in human trophoblast culture. Other groups have found that the placenta becomes progressively more sensitive to glucorticoids towards the end of pregnancy

(Speirs, Seckl, & Brown, 2004; Thompson, Han, & Yang, 2002); therefore MDR1/P-gp regulation may be dependent on the period of gestation. Cortisol regulation of MDR1/P-gp may be limited to late gestation, when maternal cortisol levels are drastically elevated and the placenta is most sensitive to glucocorticoids.

Other groups have suggested that cortisol effects syncytialization of trophoblast cells in culture (J. Challis, personal communication). We wanted to ensure that cortisol was not altering syncytialization of trophoblast cells in culture and potentially causing an effect on MDR1/P-gp. A higher concentration of cortisol was used and treatment started from 24 hours, when cells were not yet completely syncytialized. Our results show that cortisol did not affect syncytialization of trophoblast cells in culture, using Haemotoxylin staining of the nucleus. Therefore, the decrease in MDR1/P-gp produced by cortisol is most likely attributed to regulation, rather than a disruption in syncytialization.

We found that cortisol down-regulated MDR1/P-gp in human syncytiotrophoblast, however, the mechanism of this regulation is unknown. One possible explanation may be the regulation of MDR1/P-gp cortisol through the GR. The GR is a nuclear receptor that is in the placenta (Mark *et al.*, 2009)(Wong & Burton, 1974). The *Mdr1b* gene promoter is known to contain a GRE in the mouse (Cohen *et al.*, 1991), where the GR can potentially homodimerize and bind to directly activate gene transcription. Furthermore, the GR can also “cross-talk” signal though the AP-1 element (Gottlicher, Heck, & Herrlich, 1998), which has been found in the human *MDR1* promoter, and can form complexes with c-Jun and c-Fos proteins. GR has been shown to activate or repress AP-1 activity, based on if AP-1 is in complex with c-Jun/c-Jun or c-Jun/c-Fos (Diamond *et al.*, 1990). Dexamethasone is a synthetic glucocorticoid and a known agonist of the GR (Koenen *et al.*, 2007). In our study, dexamethasone did not appear to regulate MDR1/P-gp, providing evidence that cortisol may not regulate MDR1/P-gp through the GR. To investigate the GR further, we also used RU486, which is an antagonist of the receptor (Beck *et al.*, 1993). When cells were co-incubated with cortisol and RU486, the inhibitory effect of cortisol on MDR1/P-gp was not reversed. This implies that cortisol's effect does not occur through the GR.

Cortisol can also bind to the mineralocorticoid receptor (MR). Spironolactone, a competitive antagonist of the MR (Delyani, 2000), was co-incubated with cortisol to determine if it would interfere with cortisol's effects on MDR1/P-gp. There was variation in MDR1/P-gp levels, however, in both cortisol and a combination of cortisol and spironolactone, protein levels were lower, compared to control. In future studies, this experiment must be repeated in order to better understand the potential MR's involvement in the regulation of MDR1/P-gp by cortisol.

Orphan nuclear receptors are also mechanisms by which cortisol may regulate MDR1/P-gp in placental trophoblast cells. Orphan nuclear receptors are located in the cytoplasm and when bound to a ligand, move into the nucleus to regulate gene transcription of target genes. Cortisol can bind to the orphan nuclear receptor, pregnane xenobiotic receptor (PXR) (Schuster & Langer, 2005). PXR is known to regulate MDR1/P-gp in the brain endothelium of other species (Bauer *et al.*, 2004; Narang *et al.*, 2008; Ott, Fricker, & Bauer, 2009; Synold, Dussault, & Forman, 2001), however whether PXR is expressed in the placenta is yet to be determined.

Glucocorticoids are also ligands for another orphan nuclear receptor, the constitutive androsterone receptor (CAR) (Pascussi *et al.*, 2000). CAR and MDR1/P-gp expression positively correlate in other tissues (Burk *et al.*, 2005) and in the mouse small intestine, CAR activation upregulated the *Mdr1a* isoform but did not affect the *Mdr1b* isoform (Maglich *et al.*, 2002). It has yet to be established whether CAR is present in the placenta. Furthermore, there is evidence of cross-talk between the GR, PXR, and CAR in regulating metabolizing enzyme (C. Chen, Staudinger, & Klaassen, 2003; J. D. Schuetz *et al.*, 1996), further complicating the potential mechanistic pathway involved in cortisol regulation of MDR1/P-gp. Cortisol may also have non-receptorial effects on MDR1/P-gp, for example, by altering the shuttling of the inactive protein to the membrane where it is functional. In future studies, the PXR and CAR should be studied as potential mechanisms by which cortisol regulate MDR1/P-gp and non-receptorial regulation should also be investigated.

We found that the inhibition of MDR1/P-gp levels, potentially regulated by cortisol showed a tendency for decreased transport but did not translate to a significant change in transport activity. This disconnect between protein levels and active protein has also been found in other studies (Vahakangas & Myllynen, 2009; Petropoulos *et al.*, 2010). For example, dexamethasone exposure in late gestation significantly increased Mdr1/P-gp in the mouse placenta; however it did not change the ‘fetal-unit’ tritiated digoxin accumulation (Petropoulos *et al.*, 2010). This discrepancy between protein and function could be due to the location of the protein measured. In our present study MDR1/P-gp levels were measured from whole cell extract, yet, functional protein is only present at the cell membrane. Therefore, cortisol may decrease MDR1/P-gp in the whole cell; however, this protein may not be integrated into the cell membrane to become active. In future studies, MDR1/P-gp must be measured in membrane fractions, after incubation with cortisol.

3.2.4 Steroid Regulation of BCRP

Estrogen has been implicated in the physiological up-regulation of BCRP in the human placenta and increases towards the end of pregnancy in maternal serum. However, the literature regarding estrogen regulation of BCRP in cell lines and the human placenta has been conflicting. It has been found that estrogen decreases BCRP in estrogen receptor alpha (ERα) positive cancer cell lines (Imai *et al.*, 2005) and the BeWo cell line (H. Wang *et al.*, 2006). On the other hand, estrogen upregulated BCRP in the ERα positive MCF-7 breast cancer cell line (Zhang *et al.*, 2006) and in human trophoblast cells (Evseenko *et al.*, 2007). Furthermore, an ERE was identified in the *BCRP* promoter region (Ee *et al.*, 2004). We found that estrogen did not have a significant effect on BCRP in human trophoblast cells in our study. The inconsistency between our study and the findings of other groups could be attributed to the fact that BCRP regulation could be cell line or tissue dependent. As previously discussed, Evseenko, Paxton and Keelan (2007) treated cells trophoblast cells after 24 hours, when cells were not completely syncytialized, which could possibly account for the differences between their findings and ours.

Progesterone increases towards the end of pregnancy in humans and has been implicated in the regulation of BCRP. In a previous study, it was found that progesterone upregulated BCRP in the BeWo cell line, through the progesterone receptor B (PRB), but not through the progesterone receptor A (PRA) (H. Wang *et al.*, 2008). Conversely, Kalabis *et al.* (2007) found that progesterone treatment of pregnant mice did not change Bcrp1 levels in the placenta (Kalabis *et al.*, 2007). Furthermore, progesterone did not change BCRP levels in human trophoblast culture, at 48 hours at 100 nM (Evseenko *et al.*, 2007). The progesterone receptor (PR) is expressed in the human placenta (Cudeville *et al.*, 2000) and PRE was identified in the *BCRP* promoter, indicating a potential role of progesterone in BCRP regulation (H. Wang *et al.*, 2008). In the present study, progesterone did not have an effect of BCRP in human trophoblast culture, which is consistent with findings in the mouse placenta.

There is limited information regarding the glucocorticoid regulation of BCRP. In a few studies, dexamethasone was shown to decrease BCRP in breast cancer cell lines, *in vitro* (Elahian *et al.*, 2009; Elahian *et al.*, 2010; Honorat *et al.*, 2008). There is no information regarding the glucocorticoid regulation of BCRP in the placenta. Glucocorticoids are administered to 10% of pregnant women who are at risk of premature labour, in order to aid in fetal lung development (Koenen *et al.*, 2007), therefore women can be exposed to synthetic glucocorticoids, as well as endogenous glucocorticoids that are known to increase drastically towards the end of gestation. Our studies indicate that both cortisol and dexamethasone do not have significant effects on BCRP. Furthermore, GRE has yet to be found on the BCRP promoter.

On occasion, a double line was seen using western blot with the H-70 antibody, with one band at approximately 72 kDa and the other band at approximately 75-80kDa (**Figure 23**). Interestingly, when the syncytialized trophoblast cells were treated with cortisol this was consistently observed. A double line was also observed by others, at approximately the same molecular weights, and it has been suggested that the higher molecular weight band represents a complex-glycosylated form of the protein (Mohrmann *et al.*, 2005). Therefore, it is possible that cortisol can alter the glycosylated state of the protein and affect control in the same yet undefined manner.

Chapter 4: EXAMINATION OF THE BEWO CELL LINE AS A POTENTIAL MODEL FOR MDR1/P-GP AND BCRP REGULATION

4.1 RESULTS

4.1.1 Verification of Purity and Syncytialization of the BeWo Cell Line

The BeWo cell line is a human choriocarcinoma cell line that our laboratory was attempting to use as a primary model for the study of MDR1/P-gp regulation, in addition to human trophoblast cells. Initial studies were carried out to determine the purity of BeWo cells in culture and immunohistochemistry was performed on the BeWo cell line after 72 hours in culture, to determine if the cells aggregate, similar to cytotrophoblast fusing together to form syncytiotrophoblast in primary cell culture (**Figure 25a**). BeWo cells stained positively with cytokeratin, which stains trophoblast cells (**Figure 25b**). Vimentin, a stain for non-trophoblast cells, did not stain BeWo cells (**Figure 25c).**

4.1.2 MDR1/P-gp and BCRP in the BeWo Cell Line

BeWo cells stained positively for MDR1/P-gp after 72 hours in culture (**Figure 26b**). However, using western analysis, MDR1/P-gp could not be detected in BeWo cells, as shown in **Figure 27**. This experiment was repeated numerous times and with different methods protocols and various MDR1/P-gp antibodies (**Table 2,** pg. 33). After approximately 12 months a new batch of BeWo cells was obtained from the ATCC and MDR1/P-gp was subsequently identified using western analysis, with D-11 antibody (**Figure 28**). BCRP was identified in the BeWo cell line, using western blot with BXP-21, a monoclonal antibody raised against human BCRP (**Figure 29**).

It is controversial in the literature as to whether BeWo cells spontaneously syncytialize in culture or if forksolin is needed for syncytialization. To investigate this, BeWo cells were incubated with forskolin (20 μM), after 24 hours in culture, for an additional 72 hours. Syncytialization was investigated using an anti-desmosomal antibody, where the absence of staining around individual cells, and the presence of staining around multiple nuclei, indicate the formation of syncytialization. BeWo cells, treated with forskolin (**Figure 30d**), showed markedly reduced staining, compared to control (**Figure 30b**).

4.1.3 Forskolin Regulation of MDR1/P-gp and BCRP in the BeWo Cell Line

Other groups have suggested that increased syncytialization upregulated MDR1/P-gp in the BeWo cell line (Mark & Waddell, 2006). We investigated MDR1/P-gp regulation by forskolin, in order to potentially use the cell line as a model for MDR1/P-gp regulation. After 24 hours in culture, BeWo cells were treated for 72 hours with forskolin (20 μM), used by other research groups to induce syncytialization (Mark & Waddell, 2006). Western analysis was performed to assess the protein level of MDR1/P-gp after forskolin treatment. Forskolin (20 μM) has a tendency to increase MDR1/P-gp levels after 72 hours; however this increase was not statistically significant (**Figure 31**). The effect of forskolin on BCRP levels was also investigated. BeWo cells were treated for 72 hours with forskolin (20 μM), after 24 hours in culture, as previously described. BCRP protein levels were evaluated using Western analysis and forskolin did not have an effect after 48 hours (**Figure 32**).

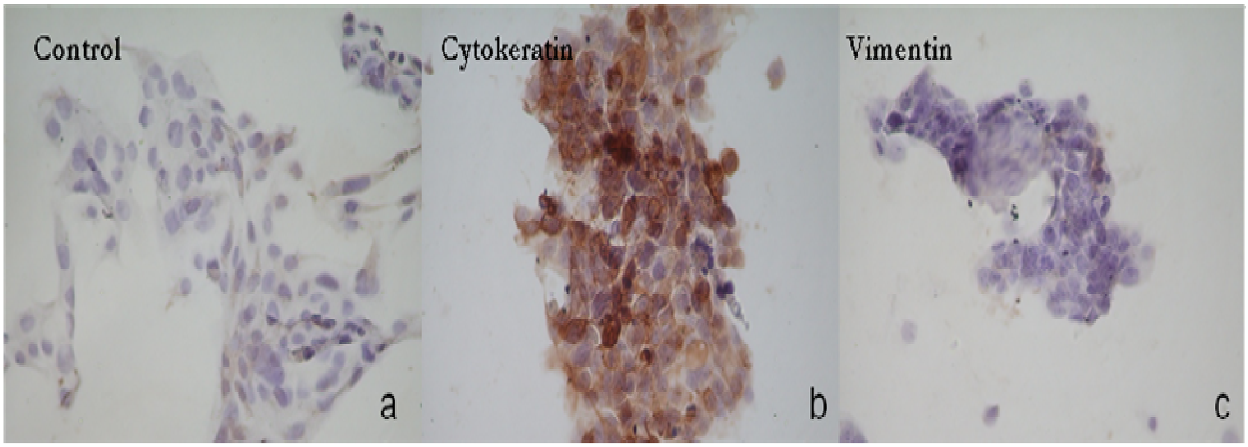

Figure 25| Verification of purity of BeWo cells after 72 hours in culture. Immunohistochemistry of the BeWo cell line after 72 hours in culture with a) control (mouse IgG), b) cytokeratin (1:3000), and c) vimentin (1:3000).

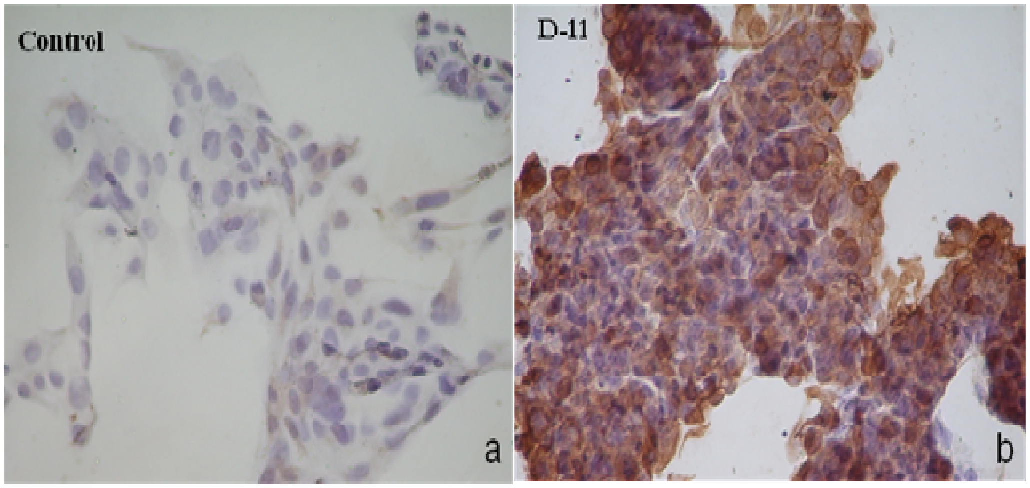

Figure 26| Immunohistochemistry of BeWo cells after 72 hours in culture. Immunohistochemistry of the BeWo cell line after 72 hours in culture with a) control (mouse IgG), b) D-11, a monoclonal anti-MDR1/P-gp antibody diluted to 1:500.

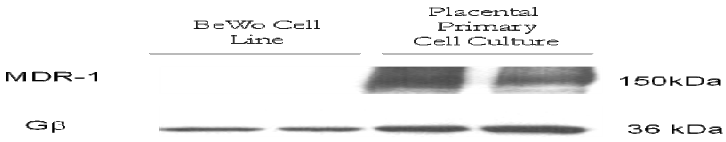

Figure 27| Typical western blot of MDR1/P-gp in BeWo cell line and placental primary cell culture. D-11, a monoclonal anti-MDR1/P-gp antibody, was used. 40 μg of protein was loaded in each lane. Gβ was used as an internal control.

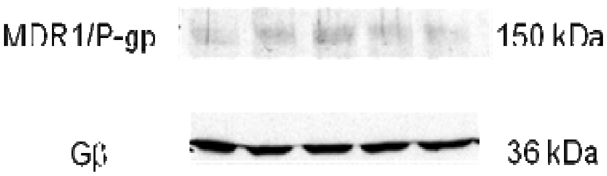

Figure 28| Typical western blot of MDR1/P-gp in the BeWo cell line. D-11, a monoclonal anti-MDR1/P-gp antibody was used. 40 μg of protein was loaded in each lane. Gβ was used as an internal control.

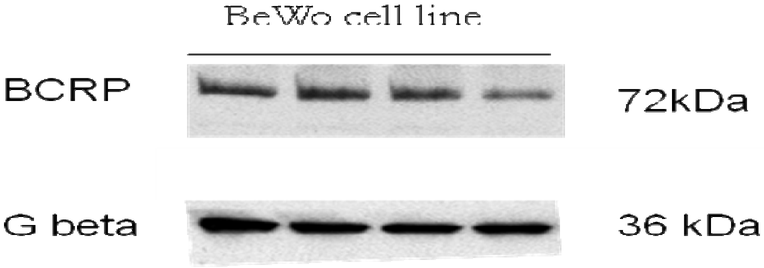

Figure 29| Typical western blot of BCRP in the BeWo cell line. BXP-21, a monoclonal anti-BCRP antibody, was used. 25 μg of protein was loaded in each lane. Gβ was used as an internal control.

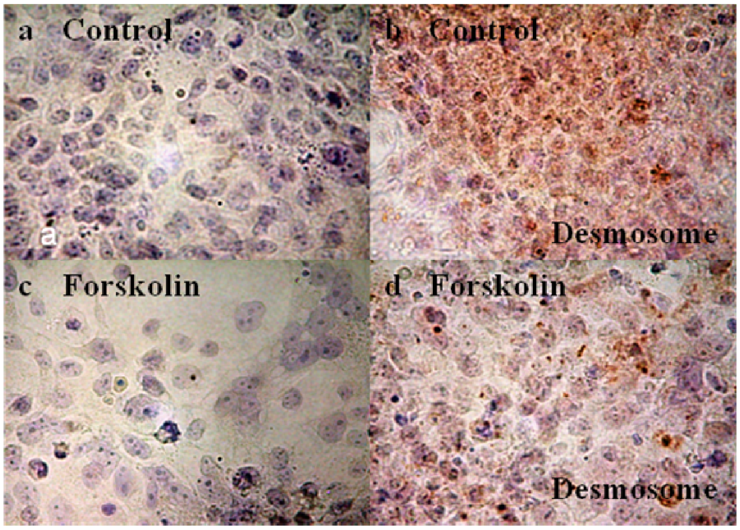

Figure 30| Immunohistochemistry of BeWo cells after treatment with forskolin. Immunohistochemistry of the BeWo cell line after 72 hours in culture with a) control (mouse IgG), b) a monoclonal anti-desmosomal cytokeratin antibody diluted to 1:500, c) pretreated with 20 µM of forskolin for 48 hours, control (mouse IgG), and d) pretreatment with 20 µM of forskolin for 48 hours, anti-desmosomal antibody diluted to 1:500.

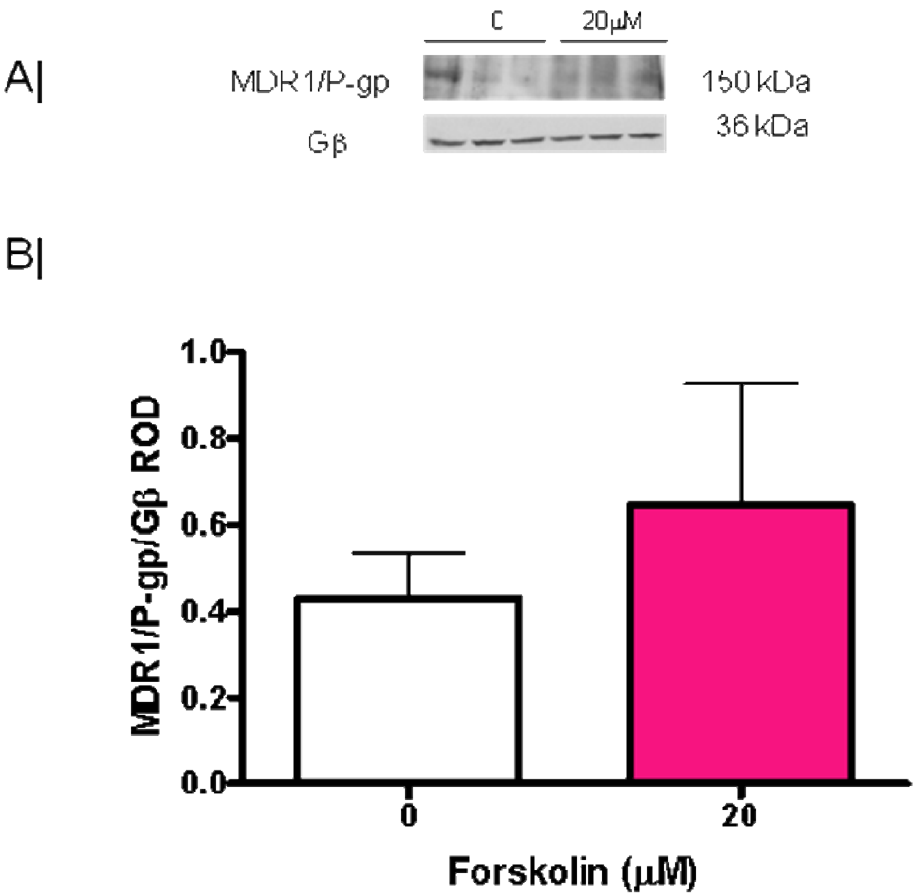

Figure 31| MDR1/P-gp levels in the BeWo cell line after 72 hours of forskolin treatment. A) A typical western blot of MDR1/P-gp and Gβ protein levels after forksolin treatment of 0 (n=6) and 20μM (n=6) for 72 hours, using D-11 antibody. **B)** Quantification of MDR1/P-gp the BeWo cell line. MDR1/P-gp was quantified after 0 and 20 μM of forskolin treatment for 72 hours. Each n number is the mean of a triplicate sample from each passage. Student t-test, Bar represents mean +/-SEM, standardized against Gβ and expressed as relative optical density (ROD).

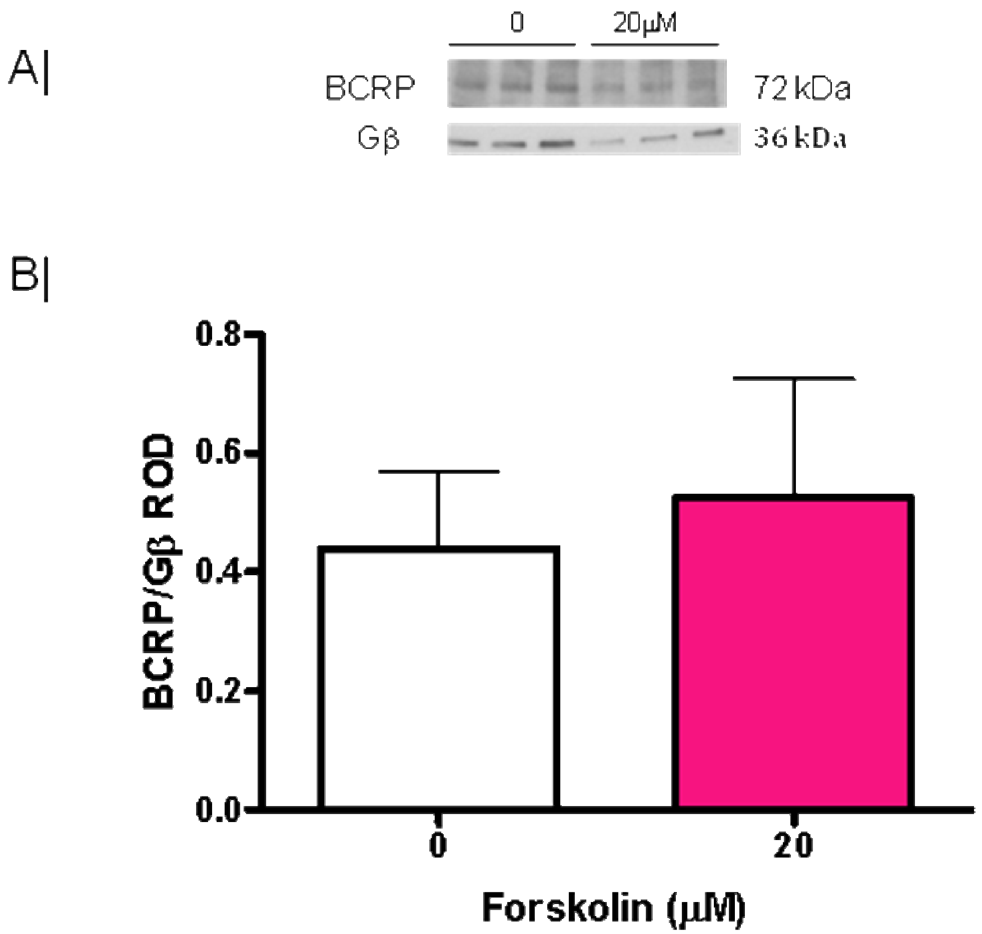

Figure 32| BCRP levels in the BeWo cell line after 72 hours of forskolin treatment. A) A typical western blot of BCRP and Gβ protein levels after forksolin treatment of 0 (n=4) and 20μM (n=4) for 72 hours, using BXP-21 antibody, a monoclonal anti-BCRP antibody. **B)** Quantification of BCRP the BeWo cell line. BCRP was quantified after 0 and 20 μM of forskolin treatment for 72 hours. Each n number is the mean of a triplicate sample from each passage. Student t-test, Bar represents mean +/-SEM, standardized against Gβ and expressed as relative optical density (ROD).

4.2 DISCUSSION

There are several limitations associated with using human placenta as an experimental model. Along with the issue of ethics, there is a limited quantity of tissue available. Additionally, the quality and quantity of cells obtained from the placenta is inconsistent and from a genetically varied population. Our laboratory attempted to use the BeWo cell line, a human choriocarcinoma cell line, as a primary model for the study of MDR1/P-gp regulation. We initially hoped to use the BeWo cell line to optimize concentration and time curves to study the regulation of MDR1/P-gp and BCRP with steroids, before carrying out studies in human trophoblast cells. Initial studies were centered on verifying purity, syncytialization, and if MDR1/P-gp was present in the cell line. BeWo cells stained positively with cytokeratin and did not stain with vimentin, which indicated that cell culture was a pure culture of trophoblast cells, similar to results with trophoblast cells.

The majority of publications propose that BeWo cells spontaneously syncytialize at low levels in culture and syncytialization is augmented by increasing intracellular cAMP, for example, by adding agents such as forskolin (Borges *et al.*, 2003; Orendi *et al.*, 2010; Rote, 2005). However, other groups argue that BeWo cells "aggregate" in culture, unless treated with an agent that increases cAMP (Coutifaris *et al.*, 1991). In the present study, cultured BeWo cells stained darkly with an antibody that detects desmosomal cell structures located in intracellular junctions, which indicates that cells were not completely syncytialized. When BeWo cells were incubated with forksolin and then stained with the demsosomal antibody, staining was markedly reduced, indicating an increase in syncytialization. Our results indicate that BeWo cells in culture do not spontaneously syncytialize, unless treated with an agent, such as forskolin, which increases intracellular levels of cAMP.

Whether or not MDR1/P-gp is present in BeWo cells is controversial. A small number of studies have identified MDR1/P-gp in the BeWo cell line using western analysis (Ushigome *et al.*, 2000; Utoguchi *et al.*, 2000), whereas, other studies have not found the protein in BeWo cells (Magnarin *et al.*, 2008). In initial studies, we detected immunoreactive MDR1/P-gp in the BeWo cell line, using IHC, but could not confirm its presence using western analysis. For months, western analysis was repeated using different passages of BeWo cells, with the D-11 and G-1 antibody. MDR1/P-gp could not be identified. The BeWo cell line was then reobtained from ATCC and MDR1/P-gp was detected, using western blot with the D-11 antibody. Our results indicate that MDR1/P-gp is inconsistently found in the BeWo cell line. In contrast, BCRP was found at high levels in BeWo cells, in the present study. This is consistent with other published studies, which have reported BCRP in the BeWo cell line (Bailey-Dell *et al.*, 2001)(Ceckova *et al.*, 2006)(Evseenko, Paxton, & Keelan, 2006a).

Mark and Waddell (2006) found that increased syncytialization of BeWo cells upregulated MDR1/P-gp (Mark & Waddell, 2006). This was investigated in our study, to determine if MDR1/P-gp could be induced and potentially used as a consistent model to study the protein, using western analysis, in the future. Forskolin treatment slightly increased MDR1/P-gp, but this was not statistically significant, in BeWo cells. The results of the western blot were still inconsistent and the increase in MDR1/P-gp was not great enough to consider the BeWo cell line as a useful model for studying the regulation of the protein. BCRP levels are highly expressed in the BeWo cell line (Evseenko, Paxton, & Keelan, 2006a); but syncytialization did not alter protein levels. At the present time we would suggest that the BeWo cell line has limited utility for studying the regulation of MDR1/P-gp but may be of use for the study of BCRP.

CHAPTER 5: SUMMARY

The purpose of this thesis was to study the physiological regulation of ABC proteins, MDR1/P-gp and BCRP, in the human placenta. Previous studies from our laboratory have shown there is a significant decrease in MDR1/P-gp in human syncytiotrophoblast with increasing gestation and slight increase in BCRP. The decrease in MDR1/P-gp could cause the fetus to become exposed to toxins, drugs, steroids and hormones from the maternal blood in the latter part of gestation but it is possible that the increase in BCRP compensates for this. Furthermore, we previously have shown that BCRP increases at term. There is limited published data on the regulation of either protein in the placenta. Estrogen and progesterone have been proposed as potential regulators of MDR1/P-gp and BCRP in cell lines, animal tissue, and human trophoblast cells. These hormones are produced by the placenta and increase towards term. Glucocorticoids are also known to increase exponentially in maternal serum at term and have been implicated in MDR1/P-gp and BCRP regulation in cell lines and animal models.

Studies in this thesis have shown that estrogen and progesterone, alone and in combination, do not have an effect on MDR1/P-gp in human trophoblast cells. Cortisol plays a potential role in down regulating MDR1/P-gp in human syncytiotrophoblast, but does not appear to regulate the protein through the GR or MR. Other potential mechanisms of regulation could be orphan nuclear receptors or by non-receptorial regulation. Transport studies indicate that cortisol does not have a significant effect on protein function. The disconnect between protein levels and protein function has been observed in other studies and could be due to the fact that in this thesis, total cell protein was measured instead of protein on the cell membrane, where MDR1/P-gp is active. In future studies, MDR1/P-gp should be measured in cell membrane extract to get a better understanding of functional protein levels. It is possible that cortisol may be increasing MDR1/P-gp levels in the total cell; however, this protein is not being incorporated into the membrane, where the protein activity is localized. Our data suggest that BCRP is not regulated by maternal steroids, namely progesterone, estrogen, and glucocorticoids, in the human syncytiotrophoblast. Evseenko, Paxton and Keelan (2007) found that estrogen upregulated BCRP in trophoblast cells; however in this study cells were treated at 24 hours (Evseenko *et al.*, 2007). We found that at 24 hours in culture, cytotrophoblast had not completely differentiated into syncytiotrophoblast, which would not be a true representation of syncytiotrophoblast in the human placenta and could account for the differences in the findings. Furthermore, Evseenko, Paxton and Keelan (2007) only used one concentration of steroid and therefore their study was extrememly limited.

There are limitations with using human placental tissue for trophoblast cell culture. Tissue was obtained from the Ottawa General Hospital from non-complicated, scheduled cesareans. The patient had to consent and the c-section had to be on time, without complication, in order to receive the tissue. Furthermore, the quality of tissue was not always consistent and it was unpredictable as to the amount of cells yielded from each culture. The population was genetically varied making results inconsistent. Therefore, in this study we were seeking an appropriate cell line to use as a model to study the regulation of MDR1/P-gp and BCRP, primarily to optimize concentration and time curves to use in trophoblast cell culture. We found that MDR1/P-gp was inconsistently present in the BeWo cell line. MDR1/P-gp could not be detected, using western blot, in the first batch of BeWo cells ordered from ATCC. This experiment was repeated using different antibodies and by different individuals in the laboratory. MDR1/P-gp was detected in the second batch of BeWo cells that were obtained at a later date from the same company; however, protein levels were still inconsistent between repeated western blots. This is an indication that the BeWo cell line might not be an appropriate model in which to study MDR1/P-gp. Due to the low levels of the protein in the BeWo cell line, Evseenko, Paxton and Keelan (2006) have also suggested that the BeWo cell line was not an appropriate model to study MDR1/P-gp (Evseenko, Paxton, & Keelan, 2006a).

Due to the limitations previously discussed, it is debatable as to whether human cell culture is a worthwhile model to study MDR1/P-gp and BCRP regulation. In regards to MDR1/P-gp, levels are higher in placental tissue from earlier gestation; however, access to such tissue is limited by availability and size of tissue would limit the studies that could be carried out. Other laboratories have used a BeWoMDR cell line, a virally transduced daughter line with elevated levels of MDR1/P-gp (Mark & Waddell, 2006). In future studies, the BeWoMDR cell line might be a better alternative to studying MDR1/P-gp, due to the increased level of protein.

In conclusion, we found that estrogen and progesterone do not have an effect on ABC transporters, MDR1/P-gp and BCRP in trophoblast cells. Cortisol does not regulate BCRP, but significantly inhibits MDR1/P-gp levels in human syncytiotrophoblast. Through this project we have made contributions concerning the regulation of MDR1/P-gp and BCRP in the human placenta.

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