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Dorothy Yeboah

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Cellular and Molecular Medicine)

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Expression and Regulation of Breast Cancer Resistance Protein in the Human Placenta and Fetal Membranes

TITRE DE LA THÈSE / TITLE OF THESIS

Dr. W. Gibb

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

Dr. A. Basak

Dr. G. Cooke

Gary W. Slater

Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

Expression and Regulation of Breast Cancer Resistance Protein in the Human Placenta and Fetal Membranes.

By

Dorothy Yeboah

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

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Abstract

Breast Cancer Resistance Protein, BCRP, is highly expressed in many different tumor tissues conferring multi-drug resistance against many chemotherapy drugs. BCRP has also been reported to be present in normal tissues including the human placenta during pregnancy. It is believed that in the placenta, BCRP controls the levels of toxins, drugs and xenobiotics that may cross maternal circulation into fetal circulation.

The expression of BCRP was examined in the human placenta and in placental membranes (amnion and chorion leave) and attached decidua. In addition, the effect of cytokines and hypoxia on BCRP expression in placental cells was examined in vitro.

BCRP was found to be highly expressed in the placenta throughout pregnancy as well as in the amnion, chorion laeve and attached decidua. Our data suggest that increased cytokine expression and reduced oxygen levels may not have any effect on BCRP mRNA or protein levels in the placental syncytiotrophoblast cells. This may suggest that BCRP is stably expressed in the placenta, even under adverse conditions, and may imply that activity of this transporter protein is essential for normal placental function.

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List of Abbreviations

ABC	ATP Binding Cassette
AIDS	Acquired Immune Deficiency
ALDP	Adrenoleucodystrophy Protein
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
ATP	Adenosin Triphosphate
BCRP	Breast Cancer Resistance Protein
BPA	Bisphenol A
CFTR	Cystic fibrosis transmembrane conductance regulator
CI1033	
DHEAS	Dehydroepiandrosterone sulphate
DMEM	Debaco Modified Eagle Medium
DNA	Deoxyribonucleic acid
DSP	Dithiobis(succinimidyl)propionate
ELISA	Enzyme-linked immunosorbent assay
ERα	Estrogen Receptor alpha
ERE	Estrogen Response Element
FBS	Fetal Bovine Serum
Fbv	Fetal blood vessel
FTC	Fumitremorgin C
Gβ	Beta subunit of G-protein
Gln	Glutamine
Gly	Glycine
GSH	Glucothione
HEK	Human Embryonic Kidney
HIF1	Hypoxia Inducible Factor 1
HIV	Human Immune deficient
IL-1β	Interleukin-1-beta
IL-6	Interleukin-6
Ile	Isoleucine
JAR cells	

Lue	Leucine
Lys	Lysine
MCF7/AdrVp	MCF-7 cells selected for resistance to the anthracycline doxorubicin in the presence of verapamil
MDR1	Multi-drug resistance protein 1
Met	Methionine
mRNA	messenger Ribonucleic Acid
MRP	Multi-drug resistance associated proteins.
MSD	Membrane spanning domain
MXR	Mitoxantrone resistant
NBD	Nucleotide binding domain
P-gp	P-glycoproteins
PhIP	2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
PKA	Protein Kinase A
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SN-38	Topoisomerase (topo) I poison 7-ethyl-10-hydroxycamptothecin
SP	Side population cells
Syn	Syncytiotrophoblast
TAG	temoxifen derivative
TAP	Transportor associated with Antigen Presentation
Thr	Threonine
TKI	Tyrosine Kinase Inhibitor
TNFα	Tumor Necrosis Factor alpha
Tyr	Tyrosine
Val	Valine

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Chapter 1

1 Introduction

Drug use during pregnancy may be inevitable due to treatment of a chronic disease such as cancer, HIV/AIDS, diabetes and hypertension or lack of knowledge of the pregnancy, especially during the first trimester, as an important proportion of pregnancies are unplanned (Koren *et al.*, 1998). It has been well documented that maternal drug intake during pregnancy can have adverse effects on the developing fetus.

Fetal alcohol exposure is the leading cause of mental retardation in the United States (Abel & Sokol, 1986). Studies show that even low levels can have a severe impact on fetal development (Eckardt *et al.*, 1998;Olsen & Tuntiseranee, 1995). Prescription drugs used to treat epilepsy, diabetes, cancer and other chronic diseases have been reported to cause severe developmental defects (Berglund *et al.*, 1984;Bertollini *et al.*, 1987;El Beitune & Duarte, 2006). The discovery of ATP binding cassette (ABC), transporters that have the ability to bind to, and extrude, many different drugs from placental barrier cells has resulted in the investigation of the expression, regulation, function and physiological roles of these proteins in the human placenta. This thesis is focused on one such transporter that is highly expressed in the human placenta, namely, Breast Cancer Resistance Protein (BCRP).

1.1 ABC proteins; General overview

ABC proteins are a super family of membrane spanning transporters or cellular pumps that; a) mediate active efflux of compounds out of cells, b) limit the net uptake of compounds, c) including nutrients, by cells and d) reduce the net penetration of compounds at blood tissue barriers. This family is one of the largest and most diverse groups of transporters with 49 members, 17 of which have been implicated in disease (Dean *et al.*, 2001;Dean & Annilo, 2005;Gottesman & Ambudkar, 2001). For instance, ABCC7 also known as the cystic fibrosis transmembrane conductance regulator, CFTR, is an ion channel and a member of the ABC family, which has been linked to Cystic Fibrosis (Riordan *et al.*, 1989). ABCC2 or multi-drug resistance associated protein 2 (MRP2) has also been linked with Dubin-Johnson syndrome (Kartenbeck *et al.*, 1996), a genetic disorder characterized by chronic conjugated hyperbilirubinemia and deficient hepatobilliary transport of anion conjugates (Dubin & Johnson, 1956). Mutation in ABCA4 has been associated with age related macular degeneration and Startgardt disease, a disease characterized by juvenile to early adult onset macular dystrophy with loss of central vision (Allikmets, 1997;Allikmets *et al.*, 1997). This indicates the importance of ABC proteins in normal cellular function.

ABC proteins are found in all organisms from bacteria to mammals and are responsible for the translocation of a wide range of compounds. Compounds are transported across intracellular or plasma membrane of cells against their concentration gradient using energy from ATP hydrolysis (Borst & Elferink, 2002). These compounds

include; drugs, toxins, metabolic products, endogenous compounds such as lipids, peptides, nucleotides, ions, organic-anions, bile acids, polysaccharides and sterols (Klein *et al.*, 1992;Schinkel & Jonker, 2003). All 49 members of the ABC family have a conserved ATP binding domain and nucleotide and protein sequence homology.

The conserved hydrophilic ATP binding domain or the Nucleotide Binding Domain, NBD, has two motifs known as Walker A and Walker B which contain amino-acid residues that interact with the β-phosphate of ATP and Mg^{2+} (Hung *et al.*, 1998;Sharom *et al.*, 1999;Walker *et al.*, 1982). Located between Walker A and B is the signature motif or C motif. The C motif is believed to participate in ATP binding and hydrolysis although its precise function is not presently clear (Leslie *et al.*, 2005). The C motif distinguishes ABC proteins from other ATP binding proteins (Krishnamurthy & Schuetz, 2006). Another typical structural domain of ABC transporters is the hydrophobic Membrane Spanning Domain, MSD, composed of four to eight trans-membrane α-helices (Krishnamurthy & Schuetz, 2006). These two domains make up the core of ABC transporter proteins. Most of mammalian ABC transporters have cycle repeated halves of the two domains linked by a polypeptide sequence. That is, a sequence of MSD-NBD-MSD-NBD (see Figure 1.1)(Ambudkar *et al.*, 2003;Borst & Elferink, 2002;Klein *et al.*, 1992;Schinkel & Jonker, 2003). ABCC or MRP proteins have an extra MSD at the amino terminal of the protein (Bakos & Homolya, 2007;Cole *et al.*, 1992).

Crystal structure studies of the NBD of a few bacteria ABC proteins suggest that these proteins undergo a conformational change upon binding ATP that forces substrate

across the membrane (Locher *et al.*, 2002;Chang, 2007;Chang & Roth, 2001). Other domains may be present on ABC proteins. These are collectively called accessory domains, as in some cases their absence has shown no effect on protein functions. However, they may have regulatory functions on the transporter (Biemans-Oldehinkel *et al.*, 2006).

Figure 1.1

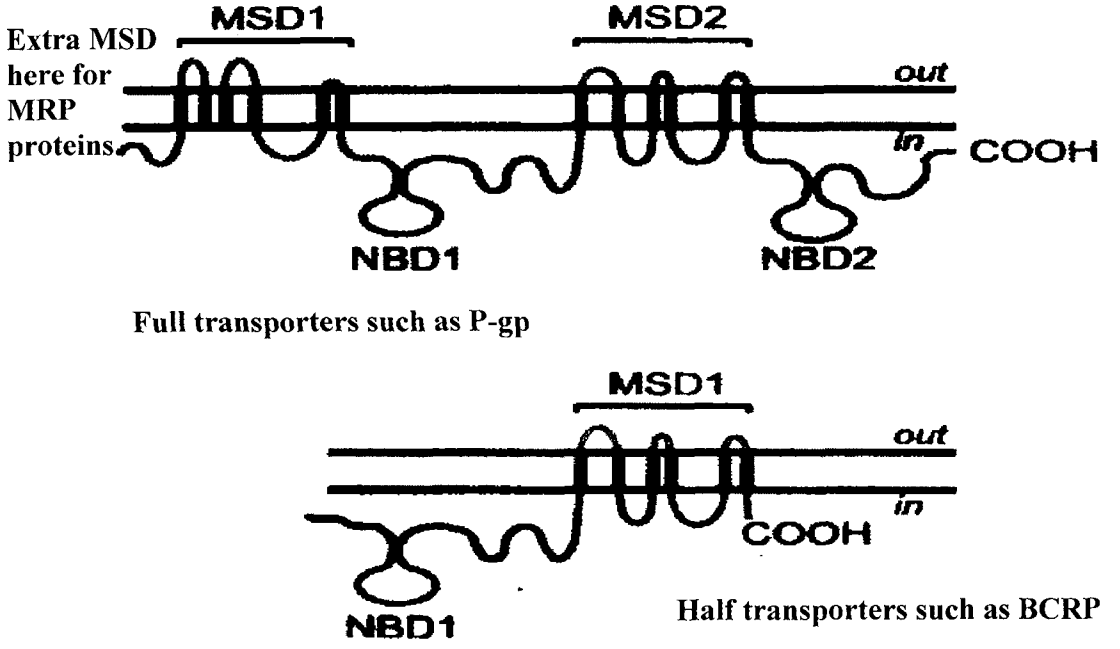

Figure 1.1: Proposed model of the arrangement of ABC protein in cell membranes

Full ABC transporters have 2 cycle repeats of the Membrane Spanning Domain (MSD) and the Nucleotide Binding domain (NBD). Half ABC transporters have one of each domain in a reverse arrangement of a full transporter. Image was taken from Leslie et al (2005) with modifications.

These domains include; an extra-cytoplasmic domain which is usually a substrate binding domain in many ABC uptake systems in prokaryotes (Biemans-Oldehinkel *et al.*, 2006), membrane embedded domains which range from an extra transmembrane segment to an extra MSD as in the case of MRP1 (see figure 1.1) (Westlake *et al.*, 2003) and cytosolic regulatory domains, which includes the R-domain of CFTR proteins and the L0 linker of MRP1 proteins (Rich *et al.*, 1991). The R-domain of CFTR contains phosphorylation sites for PKA. Phosphorylation by PKA plus ATP binding and hydrolysis causes this chloride channel to open. The L0 linker domain connects the extra MSD of MRP1 proteins to the rest of the protein.

Physiologically, ABC proteins protect the body from the deleterious effects of toxin accumulation and also regulate intracellular levels of compounds needed for normal cell function (Dean *et al.*, 2001;Schmitz & Kaminski, 2001). The 49 members of the ABC proteins have been organized into 7 subgroups named A to G. This sub-grouping is based on gene structure, amino acid sequence homology and phylogenic analysis (Dean & Annilo, 2005). The most recent member of the ABC family to be discovered, BCRP, is mainly expressed in epithelial barrier cells and is believed to regulate tissue penetration of substrates and extrude a wide variety of compounds such as anticancer agents including mitoxantrone, topotecan and flavopiridol, to mention a few (Borst & Elferink, 2002;Doyle & Ross, 2003;Schinkel & Jonker, 2003).

1.1.1 BCRP and the ABCG subgroup

BCRP was first isolated from a human doxorubicin (anticancer drug) resistant breast cancer cell line, MCF-7/AdrVp(Doyle *et al.*, 1998). This is the origin of its name, but there is no evidence to suggest that it is specific for, or abundantly expressed in breast cancer tissues. It is a 72kDa protein, under reducing conditions, with 655 amino acids (Kage *et al.*, 2002;Maliepaard *et al.*, 2001;Rocchi *et al.*, 2000). Others have also named it mitoxantrone resistance protein (MXR) because of its role in the resistance to this anti-cancer drug (Miyake *et al.*, 1999).

BCRP belongs to the White or the ABCG subgroup (Allikmets *et al.*, 1998). The first member of this group, ABCG1, was identified as the human homolog of the Drosophila white gene, sharing about 60% amino acids of the Drosophila protein (Croop *et al.*, 1997). BCRP is the second member of this subgroup, which also contains the Drosophila brown and scarlet protein genes. It was therefore named ABCG2 by the Human Genome Nomenclature committee. The other members of this group are ABCG4, ABCG5, ABCG6, ABCG7 and ABCG8. All members of the ABCG subgroup are half transporters. They each have a single NBD and a single MSD (see figure 1.1) (Brangi *et al.*, 1999). Also these proteins have a unique domain organization in that their NBD precedes the MSD. This arrangement is opposite in the full transporter proteins (Mao *et al.*, 2004) (see Figure 1). These half transporters must dimerize to become functional (Scheffer *et al.*, 2002;Rocchi *et al.*, 2000;Maliepaard *et al.*, 2001;Kage *et al.*, 2002).

The Drosophila white protein dimerizes with brown or scarlet proteins to transport tryptophan and guanine for eye pigment formation (Ewart & Howells, 1998). ABCG5 and ABCG8, which have been implicated in cholesterol and plant sterol transport, have been reported to form functional heterodimers essential for biliary cholesterol excretion (Graf *et al.*, 2003). It is not yet clear whether ABCG1 forms a homodimer or a heterodimer in its function of regulating cellular lipid homeostasis in macrophages (Klucken *et al.*, 2000). The functions of ABCG4, ABCG6 and ABCG7 are unknown as yet (Mao *et al.*, 2004).

Heterologous overexpression of BCRP in mammalian or insect cells produced a functional drug transporter. Therefore, it is very likely that BCRP functions as a homodimer (Ozvegy *et al.*, 2001;Honjo *et al.*, 2001). Also, studies showing a partial dominant negative effect of mutant BCRP on wild-type BCRP function suggest that the protein functions as a homodimer (Kage *et al.*, 2002). In this study an amino acid substitution, Leu to Pro, at position 554 in the fifth transmemrane α-helix resulted in a partial reduction of resistance to SN-38, a topoisomerase 1 inhibitor, and mitoxantrone when cotransfected with wild type BCRP. Both compounds are anti-cancer agents and known substrates of BCRP.

Litman et al have reported a higher molecular weight protein (180kDa) following treatment with a chemical cross-linking agent (Litman *et al.*, 2002). This protein complex is larger than that observed by Kage et al who described a 140kDa protein complex after running SDS-PAGE under non-reducing conditions (Kage *et al.*, 2002). Recently, studies

by Xu et al, using sucrose density gradient sedimentation, and non-denaturing gel electrophoresis demonstrated that BCRP might form homotetramers (Xu *et al.*, 2004). These data strongly suggest that BCRP functions as a complex with itself.

Surprisingly, recent studies by Mitomo et al, found that BCRP maintained full methotrexate transport activity in the presence of 10mM of β-mecaptoethanol implying that disulfide bonds may not be important in the functional form of BCRP since this concentration of β-mecaptoethanol is enough to break the disulphide bonds between monomers (Mitomo *et al.*, 2003). It appears rather that the GXXXG motif in the first transmembrane segment of BCRP may have an important role to play in dimerization, oligomerization or proper folding of the protein. This motif is believed to be involved in the dimerization of other proteins including glycoprotein A and human carbonic anhydrase. Mutations in this region results in a reduction in BCRP mediated drug transport (Polgar *et al.*, 2004).

BCRP is the only member of the ABCG subgroup that has been shown to be expressed in the human placenta. Other members such as ABCG5 and ABCG8 are expressed in the liver and intestines (Schmitz & Kaminski, 2001). Half transporter proteins are generally localized to intracellular membranes, as in the case of TAP1/TAP2 on endoplasmic reticulum, ALD on peroxisomes, and M-ABC1 and ABC7 on mitochondria (Allikmets *et al.*, 1999;Hogue *et al.*, 1999;Kok *et al.*, 1995;Townsend & Trowsdale, 1993;Wang *et al.*, 1998). However, BCRP has been reported to be mainly located on the plasma membrane of cells which is typical for full ABC transporters such

as MRP and P-gp (Dean *et al.*, 2001;Litman *et al.*, 2000;Rocchi *et al.*, 2000). On a few occasions BCRP has been found in intracellular membranes (Rocchi *et al.*, 2000;Rajagopal & Simon, 2003).

A mouse homologue, bcrp1, with 81% identity to the human protein was discovered in mouse fibroblast cells selected for resistance to the anti-cancer drugs: mitoxantrone, doxorubicine and topotecan. The mouse bcrp1 extrudes compounds in an energy dependent manner just like its human counterpart (Allen *et al.*, 1999). The human BCRP is located on chromosome 4q22 and is highly amplified in cells over expressing BCRP (Allikmets *et al.*, 1998;Knutsen *et al.*, 2000;Miyake *et al.*, 1999).

Studies have shown that a naturally occurring single nucleotide polymorphism within the coding region of BCRP affects substrate specificity (Honjo *et al.*, 2002). It was discovered that while some cell lines overexpressing BCRP conferred high resistance to anthracyclines, other did not. Also, topotecan resistance was high in some cell lines but low in others. Furthermore, while some cell-lines were resistant to rhodamine 123, others were not. In all cell-lines however, mitoxantrone resistance was consistently high (Allen & Schinkel, 2002).

cDNA sequence analysis revealed that Arg at position 482 of the BCRP sequence was replaced by either Thr or Gly in some of these cell lines (Honjo *et al.*, 2002;Robey *et al.*, 2001b). These mutant proteins were able to transport anthracycline, doxorubicin or rhodamine but the wild type BCRP was not (Allen *et al.*, 1999;Janvilisri *et al.*,

2005;Sarkadi *et al.*, 2004). This suggests that Arg^{482}, which is located near the amino terminal of the third putative transmembrane α-helix, may participate directly or indirectly in substrate binding (Allen & Schinkel, 2002). If this is the case then charged amino acids in the MSD may be essential for substrate recognition (Mao & Unadkat, 2005). This discovery has prompted studies of the effects of point mutations on BCRP function.

A study in which amino acids in the transmembrane regions of the protein were substituted to observe the effect of these mutations on BCRP substrate recognition revealed that substitution of Gln 446 which is proposed to be within, or proximal to, the second transmembrane α-helix resulted in loss of resistance to SN-38 and mitoxantrone. Another substitution of Asn at position 557, which is hypothesized to be located within or proximal to the fifth transmembrane α-helix, with Asp resulted in loss of resistance to SN-38 (Miwa *et al.*, 2003). These data strongly suggest that the membrane-spanning domain of BCRP plays a significant role in substrate recognition. It is possible that substrates interact directly with amino acids in this domain. On the other hand, amino acid substitutions may alter the helical interactions in the membrane-spanning domain, which may affect the substrate recognition site of the protein (Mao & Unadkat, 2005).

40 synonymous and non-synonymous single nucleotide polymorphisms have been identified in the promoter, exon and intron sequence in 90 different ethnic groups (Zamber *et al.*, 2003). This leads to changes in drug sensitivity, transport activities and interferes with protein expression (Mizuarai *et al.*, 2004). For instance Val12Met and

Gln141Lys occur in 30-60% of Asian population but only occur in 5-10% of Caucasian and African-American populations. 39-50% of a Japanese population is heterozygous for Gln141Lys while 7% is homozygous for the same variant. In a Chinese population 60% is heterozygous for the same variant (Imai *et al.*, 2002a;Zamber *et al.*, 2003).

While some studies reported the expression level and drug resistance properties of the Val12Met variant of BCRP are similar to the wild type protein, others reported that, although the expression levels of both the Val12Met and Gln141Lys variants were comparable to the wild type, significantly lower levels of drug resistance relative to the wild type protein were observed (Imai *et al.*, 2002a;Mizuarai *et al.*, 2004). A recent clinical trial found that the Gln141Lys polymorphism was associated with diflomotecan (a BCRP substrate) disposition. In this study 5 patients with heterozygous alleles for the variant had plasma levels about 3-fold higher than those in 15 patients with wild type alleles following intravenous drug administration (Sparreboom *et al.*, 2004).
Other variants are less frequent. For example, Asn590Tyr occurs in approximately 1.5% of Caucasians and Ile206Leu is unique to Hispanic populations (Zamber *et al.*, 2003;Imai *et al.*, 2002a;Honjo *et al.*, 2002).

1.1.2 Substrate specificity of BCRP.

An estimated 40% of all human cancers develop resistance to many different chemotherapy drugs (Sauna & Ambudkar, 2007). During the course of treatment the drug resistance phenotype appears gradually and increases as tissues become less and less responsive to repeated treatment (Imai *et al.*, 2002b). It has also been reported that some

tumor tissues show intrinsic resistance where there is no response to the drug upon the initial exposure (Hooijberg *et al.*, 2006). In studies with cancer cell lines multi-drug resistance is associated with ATP dependent increases in cellular drug extrusion (Ambudkar *et al.*, 1999).

To date three ABC proteins have been associated with multi-drug resistance. These include BCRP, P-glycoprotein (P-gp) or ABCB1 and MRP1 or ABCC1. Of the ABCC sub-family MRP2, MRP3, MRP4 and MRP5 are all being studied as potential drug transporters (Cole et al1992; Evers et al 1998; Kool et al 1997).

A number of studies have shown an overexpression of BCRP in different cell types that conferred multi drug-resistance (Allen *et al.*, 2002a;Allen *et al.*, 2002b;Doyle & Ross, 2003). Indeed, BCRP was isolated from cell lines that demonstrated resistance to anti-tumor agents in the absence of P-gp and MRP1 overexpression. These cells, however, were sensitive to some anti-tumor agents that are known to be substrates of P-gp and MRP1 proteins (Doyle *et al.*, 1998). These studies indicated that there were likely common substrates for these proteins.

Substrates recognized by these drug transporters sometimes do indeed overlap, For instance, all three proteins are able to transport the anticancer agents daunorubicin, doxorubicin and epirubicin. Both BCRP and MDR1 transport the anticancer drugs mitoxantrone, topotecan, bisantrene and anthracyclines. They also transport organic

compounds including rhodamine 123, BODIPY-prazosin and Hoechst 33342 (Rocchi *et al.*, 2000;Shapiro *et al.*, 1998).

There are however substrates that are specific to BCRP. BCRP specifically recognizes a spectrum of drug substrates that include flavopiridol, camptothecins (topoisomerase 1 inhibitors) such as SN-38, aza-anthrapyrazole (BBR3390), a mitroxantrone analog (Mao & Unadkat, 2005) and the experimental indolocarbazol topoisomerase 1 inhibitors NB-506 and J-107088 (Kawabata *et al.*, 2001;Robey *et al.*, 2001b;Komatani *et al.*, 2001). A recent study by Wang et al, that used the BCRP overexpressing cell line MT-4/DOX_{500} to study cytotoxicity of the human immunodeficiency virus (HIV) drugs zidovudine or azidothymidine (AZT) and lamivudine (3TC), reported a reduced level of cytotoxicity in these cells compared to the parent cell line MT-4 (Wang *et al.*, 2003). This suggests that these drugs are substrates for the transporter. Indeed, a follow up study confirmed that AZT and its metabolite, AZT 5 '-monophophate, are substrates of BCRP (Wang *et al.*, 2004a).

BCRP also transports dyes such as lysotracker green (Kim *et al.*, 2002;Robey *et al.*, 2001a;Scharenberg *et al.*, 2002). Besides drugs, dyes and xenobiotics, BCRP transports metabolic compounds including methotrexate and glutamates conjugates of methotrexate. Methotrexate is a folate antimetabolite, which is used to block the synthesis of thymidine by blocking the activity of dihydrofolate reductase. Thymidine synthesis is necessary for DNA synthesis and thus cell division. BCRP also transports folate and in folic acid deprived cultures of MCF7 cells, BCRP expression was markedly reduced.

These activities link BCRP to folic acid homeostasis in cells. (Ifergan *et al.*, 2004;Hooijberg *et al.*, 2006). Folate is required for 1-carbon transfer and is important for DNA and protein synthesis. Its role in DNA methylation, which is important for silencing gene expression (Jacob, 2000), has recently been shown to be important in fetal programming (Antony, 2007). It has long been recognized that folate is essential for normal embryogenesis and fetal development.

BCRP has also been shown to interact with and transport certain steroids. For example, it has a high affinity for estrone-3-sulphate and dehydroepiandrosterone sulphate (DHEAS) (Suzuki *et al.*, 2003;Imai *et al.*, 2003). Since the human placenta and placental or fetal membranes metabolize these steroids, BCRP may therefore be important in steroid synthesis in pregnancy (Imai *et al.*, 2003).

1.1.3 Inhibitors of BCRP

GF120918, an acridone carboxamide derivative and a very potent inhibitor of P-gp has been shown to be a very effective inhibitor of BCRP as well, with IC_{50} values of approximately 50 nM. (Allen *et al.*, 2002b;de Bruin *et al.*, 1999). These are concentrations that are tolerable to both humans and animals and have been used in vivo studies with mice to inhibit bcrp1 (Allen *et al.*, 2003;Jonker *et al.*, 2000;Kruijtzer *et al.*, 2002). Thus far, this is the only inhibitor that works effectively against both P-gp and BCRP. Other P-gp inhibitors have very little effect on BCRP activity (Allen & Schinkel, 2002).

Fumitremorgin C (FTC) a compound produced by the fungus *Aspergillus fumigatus* reverses drug resistance mediated by BCRP in BCRP expressing cells. FTC is a tremorgenic mycotoxin. At concentrations below levels that are toxic to cultured cells it inhibited BCRP function but had little effect on P-gp or MRP mediated transport (Robey *et al.*, 2001a;Rabindran *et al.*, 2000;Minderman *et al.*, 2002). Ko132 and Ko134 both analogues of FTC have also proven to be potent inhibitors of BCRP function (Allen *et al.*, 2003;van *et al.*, 2001).

A new line of anti-cancer drugs known as TKIs or Tyrosine Kinase Inhibitors, which are targeted against cellular signaling pathways, have been shown to inhibit BCRP mediated transport of topotecan and SN-38 due to competitive binding. This includes the drug CI1033 (Erlichman *et al.*, 2001). HIV protease inhibitors such as ritonavir, saquinavir and nelfinavir have also been shown to effectively reduce mitoxantrone sensitivity in HEK cells expressing BCRP. These protease inhibitors are not BCRP substrates therefore inhibition is not due to competitive binding (Gupta *et al.*, 2004). Also, the antibiotics novobiocin (Shiozawa *et al.*, 2004) and the estrogen antagonists tamoxifen and its derivatives, TAG-11 and TAG-139, have all been implicated in the reduction of BCRP mediated drug efflux activity (Sugimoto *et al.*, 2003).

1.1.4 Tissue distribution and sub-cellular localization of BCRP

Non-malignant tissues also express BCRP and other multidrug resistance ABC proteins. It is believed that in these tissues the resistance proteins function to protect cells from the accumulation of xenobiotics (Leslie *et al.*, 2005). BCRP is mostly localized to

the apical surface of epithelial cells in barrier tissues including the blood-brain barrier, liver, gut, kidney and placenta.

In the liver BCRP is found in hepatocytes and is located in the bile canalicular membrane (Chandra & Brouwer, 2004). In the small and large intestines it is located in the luminal membrane of the villous epithelial cells, where it is likely involved in the regulation of absorption and excretion (Erlichman *et al.*, 2001). In the blood brain barrier BCRP is expressed in the luminal surface of brain capillaries (Cisternino *et al.*, 2004). BCRP has also been found in the duct and lobules of breast and the endothelial cells of veins and capillaries of all of these tissues (Erlichman *et al.*, 2001;Allen & Schinkel, 2002)

High expression of BCRP mRNA has also been detected in bone marrow stem cells. Studies have shown that side population (SP) cells of murine bone marrow express high levels of bcrp1. Loss of bcrp1 expression in these cells led to cell death in bone marrow and skeletal muscle SP cells. Mdr1a/1b$^{-/-}$ mice however had a regular number of SP cells in the bone marrow or liver (Zhou *et al.*, 2001;Zhou *et al.*, 2002). Also, bcrp1 mRNA was highly expressed in mouse hematopoietic stem cells prior to differentiation (Zhou *et al.*, 2001;Krishnamurthy & Schuetz, 2005). BCRP expression has been observed in other stem cells as well. These include those found in the pancreatic islet (Lechner *et al.*, 2007), interstitial spaces of skeletal muscles (Tamaki *et al.*, 2002), the liver (Shimano *et al.*, 2003) and heart (Martin *et al.*, 2004). It has been proposed that BCRP in stem cells enables them to thrive under hypoxic conditions. Heme accumulates in cells under

conditions of low oxygen and since pheophorbide *a*, a BCRP substrate, is structurally related to porphyrin precursors in heme biosynthesis, it is believed that BCRP prevents the toxic effects of heme build up in stem cells by extruding the porphyrin precursors of heme (Krishnamurthy *et al.*, 2004).

In trying to understand the physiological function of BCRP in normal tissues bcrp1 knockout mice have been developed (Jonker *et al.*, 2002;Zhou *et al.*, 2002). These mice are viable, healthy and fertile. However, they were found to be light sensitive due to cellular accumulation of pheophorbide *a,* a metabolic product of chlorophyll. This revealed that BCRP is essential for the regulation of pheophorbide *a* levels in cells. Studies of BCRP function in the brain using bcrp1 knockout mice have shown that BCRP reduces drug penetration at this site. The knock out mice showed an increase in brain penetration of [^{14}C]PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine), a dietary carcinogen and bcrp1 substrate (Erlichman *et al.*, 2001) and an increase in brain penetration of the BCRP substrates mitoxantrone and prazosin but not vinblastine (which is a P-gp specific substrate) (van Herwaarden A.E et al 2003).

In other studies with mdr1a/1b knockout mice, treatment with the bcrp1 inhibitor GF120918 co-administered orally with topotecan, increased the bioavailability of topotecan by 6 fold. The hepatobilary excretion of intravenous administered topotecan decreased by 2 fold in the presence of orally administered GF120918. The initial plasma clearance of the drug also decreased by 2 fold (Jonker *et al.*, 2000). These studies suggest that one of the physiological roles of bcrp is most likely the regulation of tissue

distribution of drugs. Levels of bcrp1 message and transport functions are increased in mdr1a knockout mice compared to wild type mice. This upregulation of bcrp1 appears to be compensatory to the absence of mdr1 (Cisternino *et al.*, 2004).

Although the mouse has been used to study BCRP function and expression it is important to underline that the distribution of bcrp in this species is not the same as in the human. For example, very high BCRP mRNA expression levels have been found in the human placenta whereas in mouse placenta the mRNA expression levels are rather moderate (Jonker *et al.*, 2000). On the other hand, the mouse kidney shows high levels of expression while BCRP expression in the human kidney is low (Allikmets *et al.*, 1998;Doyle *et al.*, 1998;Jonker *et al.*, 2000).

The highest expression of BCRP has been found in the human placenta (Doyle *et al.*, 1998) followed by prostate, small intestine, brain, colon, liver and ovary. In the placenta BCRP is localized to the syncytiotrophoblast cells, which face the maternal circulation (Maliepaard *et al.*, 1999). The potential role of BCRP in placenta and fetal tissues in affording protection to the human fetus has yet to be investigated.

In most cells BCRP is primarily localized to plasma membrane, which is logical in view of its ability to extrude compounds. Several antibodies have confirmed this localization (Scheffer *et al.*, 2000;Erlichman *et al.*, 2001). In polarized cells BCRP mediates apical efflux of substrates indicating that BCRP is located on the apical membrane of these cells. Indeed, this has been confirmed by immunohistochemistry in

human and mouse studies and also in polarized canine and porcine epithelial cells (Jonker *et al.*, 2000). While other drug resistance proteins such as P-gp and MRP2 have a similar expression and localization pattern as BCRP, MRP1 is usually localized to the basolateral membrane of tissues (Sarkadi *et al.*, 2004;Haimeur *et al.*, 2004;Fromm, 2004). On a few occasions however, BCRP has been localized to the cytoplasm. For example in their study of the effect of folate deprivation on BCRP expression and function, Ifergan et al reported that when MCF7 cells were deprived of folate, 86% of BCRP produced was localized to the cytoplasmic compartment and 14% localization on plasma membrane (Ifergan *et al.*, 2005).

1.1.4.1 BCRP in Pregnancy

ABC transporter expression in the placenta has been shown to be important in protecting the fetus. For example, when mdr1a +/- mice were bred all fetuses of mdr1a (-/-) genotype developed cleft palate when mothers were treated with synthetic isomer of avermectin (Lankas *et al.*, 1998). Furthermore mdr1a (-/-)/mdr1b (-/-) fetuses showed higher level of drug accumulation than wild-type fetuses. Also, treatment of pregnant mice with an mdr1 inhibitor resulted in increased drug accumulation in fetuses in all genotypes (Smith *et al.*, 1999). MRP1 is also expressed at the basolateral surface of syncytiotrophoblast cells and could be involved in keeping compounds such as nutrients and hormones in the fetal compartment (Ifergan *et al.*, 2005;Hooijberg *et al.*, 2004).

BCRP has also been shown to protect the fetus by limiting the entry of drugs into the fetal circulation. When labeled topotecan was intravenously administered to pregnant mice pretreated orally with the BCRP inhibitor GF120918, radioactivity in the fetal plasma increased 3.2 fold whereas radioactivity in maternal plasma increased 1.6 fold when compared with mice that were not treated with the inhibitor (Jonker *et al.*, 2000).

In the human placenta the localization of BCRP on the syncytiotrophoblast indicates that it functions to protect the fetus and may be involved in limiting access of various compounds including, flavonoids, xenobiotics and steroids.

The inhibitory effects of polyphenols especially flavonoids such as genistein, daidzein and hesperetin on BCRP mediated drug transport has been reported (Imai *et al.*, 2004). This could be due to competitive binding as genistein and daidzein have both been shown to interact with BCRP. Flavonoids are abundant in fruits, vegetables such as soya and plant derived beverages such as tea and red wine. A normal Western diet contains several hundred milligrams of flavonoids per day. Flavonoids including daidzein and genistein have been found in the amniotic fluid early in pregnancy which implies early fetal exposure to these compounds (Engel *et al.*, 2006) and it is likely that placental BCRP is involved in limiting the access of these flavonoids to the fetus.

Xenobiotics that are substrates for BCRP, including Bisphenol A (BPA) have also been detected in fetal serum and amniotic fluid in early gestation. BPA is used in the production of polycarbonate plastics and epoxy resins and thus is present in most

containers and food packaging materials (Ikezuki *et al.*, 2002). It has been reported that even at low environmentally relevant levels BPA can cause severe neonatal and postnatal developmental defects in mice (Takai *et al.*, 2000).

BCRP may also function to protect the fetus from maternal steroids including estradiol-17β- glucuronide (Chen *et al.*, 2003) and other estrogen derivatives. As mentioned earlier, it has been reported that BCRP transports sulphated steroids including dehydroepiandrosterone sulphate and estrone sulphate (Suzuki *et al.*, 2003;Imai *et al.*, 2003). Estrone and 17β-estradiol have been shown to reduce BCRP mediated drug resistance, which could possibly be due to competitive binding (Miwa *et al.*, 2003). From mid-gestation to term the placenta produces large quantities of estrogens and progesterone (Byers *et al.*, 2007). In addition to being transported by BCRP, placental steroids also regulate BCRP expression (Wang *et al.*, 2006).

Due to the documented associations of drug treatment during pregnancy and infant morbidity and mortality, drug use is avoided during this period if possible. When its use becomes necessary physicians must take into account the potential effect(s) of the drugs on the developing fetus (Uhl *et al.*, 2003). As a result of ethical problems with drug trials on pregnant women, there is limited evidence-based information on the effect of fetal exposure to many drugs. Therefore, our understanding of the potential deleterious effects of drugs in pregnancy often relies upon studies carried out on animals (Dellicour *et al.*, 2007). The information gathered however, may be of limited value since the responses in humans may be different. Therefore, studies with human tissues such as the

placenta and fetal membranes are essential to investigate the role and regulation of drug transporters during pregnancy.

1.2 The Human Placenta

The placenta is a multi functional organ that supplies the fetus with nutrients and oxygen from the maternal circulation, regulates entry of many compounds from the maternal circulation into the fetal circulation, eliminates waste from the fetal circulation, and secretes bioactive hormones and prostaglandins which are essential for fetal growth and development (Ganapathy *et al.*, 2000).

During the 4^{th} and 5^{th} week of pregnancy the chorionic villi of the placenta begin to form and surround the entire embryonic sac up until the 3^{rd} month of pregnancy (Boyd JD and Hamilton WJ, 1970). Between this time and the 4^{th} month, the villi at the implantation site form intricate branches and develop to form the placenta while those at the opposite end degenerate to form the placental or fetal membranes. Towards term the surface area of the villi measures up to $14m^2$ providing an extensive interface for exchange between mother and fetus (Boyd JD and Hamilton WJ, 1970).

The human placenta consists principally of three cell types that originate from the trophoblast lineage. These include the syncytiotrophoblasts, which are polarized multi-nucleated epithelial cells forming the outer layer of the placental villi (Young *et al.*,

2003), cytotophoblasts which proliferate throughout gestation and are located within the villi and the extravillous trophoblast which are non proliferative cells that invade the endometrium (Kaufmann P and Burton GJ, 1994).

In the early stages of pregnancy, the placenta is mainly composed of cytotrophoblast cells, which fuse to form multinucleated syncytiotrophoblast cells as gestation progresses. The syncytiotrophoblast are the barrier cells that are in contact with maternal blood at their apical surface. The syncytiotrophoblast cells are also responsible for hormone production (Knipp *et al.*, 1999;Kaufmann P and Burton GJ, 1994). The basolateral surface of the syncytiotrophoblast is in contact with cytotrophoblast cells, stromal cells or fetal blood vessels (see figures 1.2 and 1.3). Therefore anything going from maternal to fetal circulation must cross the apical surface and basolateral surface of the syncytiotrophoblast, cytotrophoblast cells and the endothelial cells of fetal blood vessels (see figures 2 and 3) (Leslie *et al.*, 2005).

Figure 1.2

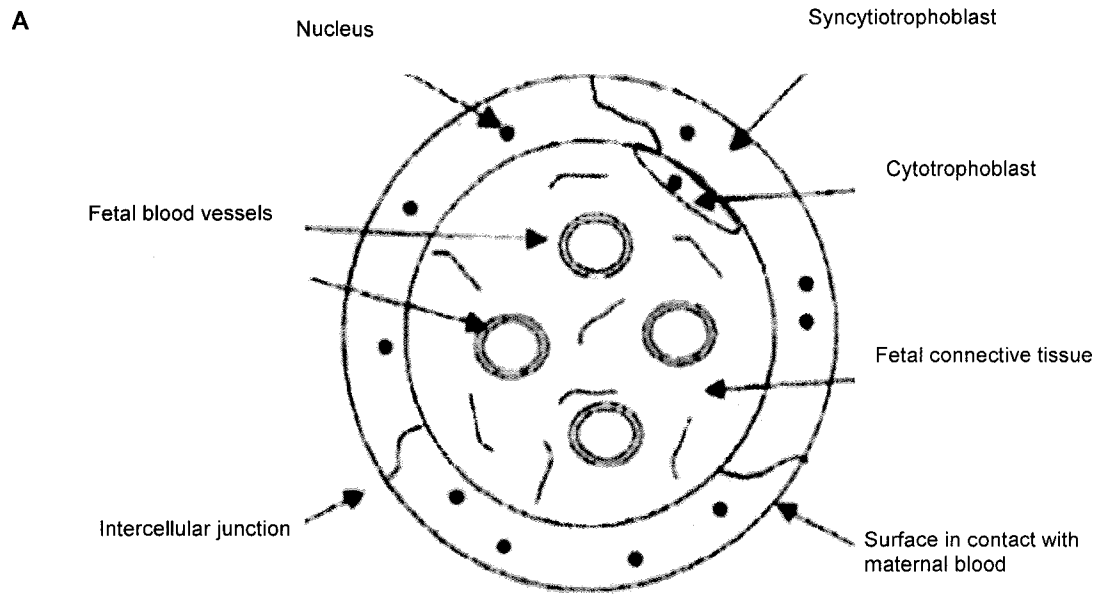

Figure 1.2A: Illustration of the arrangement of placental cells and fetal capillaries within placental microvilli.

The syncytiotrophoblasts form the barrier cells through which nutrients and gases from the maternal circulation pass to fetal capillaries located within the placental villi. Cytotrophoblast cells which fuse to form the multi nucleated syncytiotrophoblast cells are also located within the placental villi. Image was taken from Young et al (2002) with modifications.

B

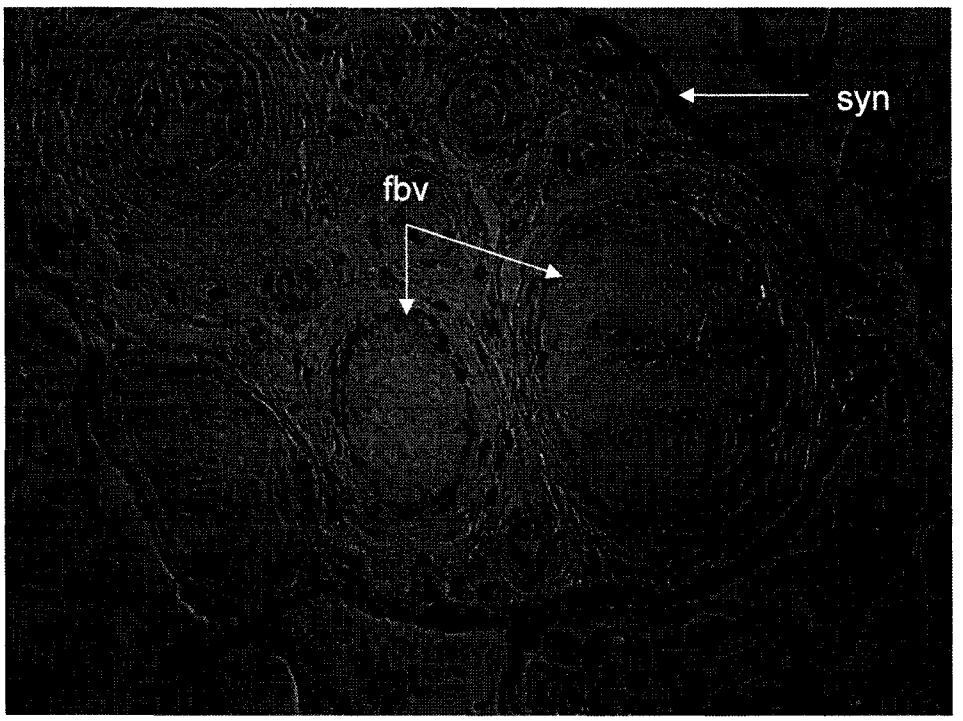

Figure 1.2B: Hematoxylin staining of human placenta tissue demonstrating the arrangement of placental cells and fetal capillaries within placental microvilli. The multinucleated syncytiotrophoblast (Syn) form the barrier cells through which nutrients and gases from maternal circulation pass to fetal blood vessels (fbv) located within the placental villi.

1.3 Human Fetal membranes

The fetal membranes are another interface between the maternal and fetal compartments. The fetal membranes comprise tissues of fetal origin; the amnion and chorion laeve, and decidua, the endometrium of pregnancy, is attached to the chorion during placenta and membrane expulsion (Rokos *et al.*, 1998). The inner membrane, the amnion, is composed of a single layer of epithelial cells that are in direct contact with the amniotic fluid and a subepithelial layer of mesenchymal cells embedded in extracellular matrix. The chorion laeve contains trophoblast cells. The decidua attached to the chorion contains stromal cells and endothelial cells of maternal blood vessels (Rokos *et al.*, 1998) (see figure 1.3). These tissues all play an important role in parturition by producing prostaglandins, cytokines and steroids which are thought to be involved in the onset of labor both at term and preterm (Bowen *et al.*, 2002;Winkler, 2003).

The fetal membranes may also have a role in the regulation of access of compounds to the fetus via the amniotic fluid. (Fukamatsu *et al.*, 1984;Woods *et al.*, 1998). There were no published findings on the presence of any multi-drug resistance transporters in these tissues until May of this year.

Figure 1.3

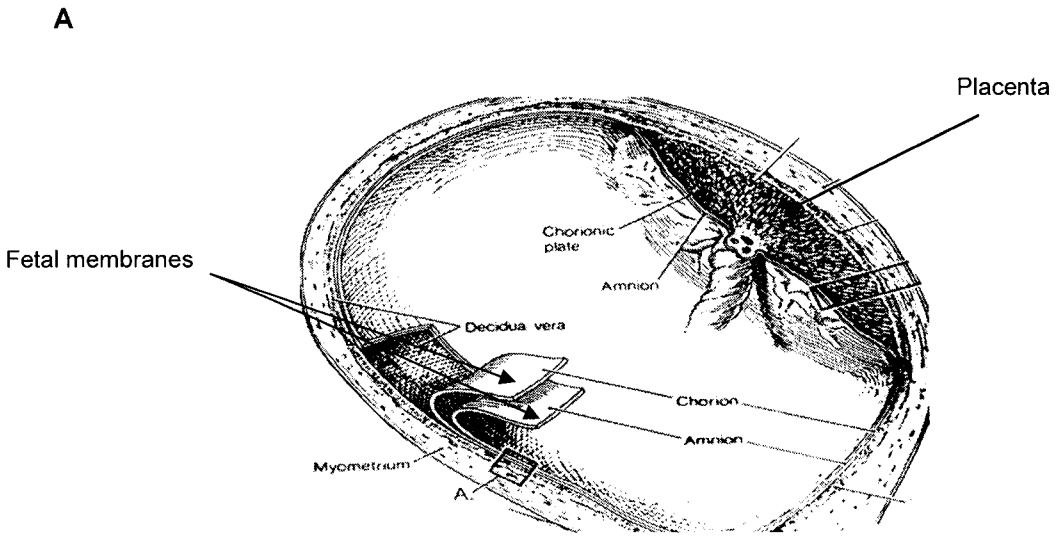

Figure 1.3A: Illustration of placenta and placental membranes arrangement in the uterus during pregnancy.

The placental membranes, amnion and chorion, separate the fetal compartment from the maternal compartment. The amnion is in direct contact with the amniotic fluid while the chorion is attached to the decidua, which is of maternal origin (Boyd JD and Hamilton WJ, 1970).

E

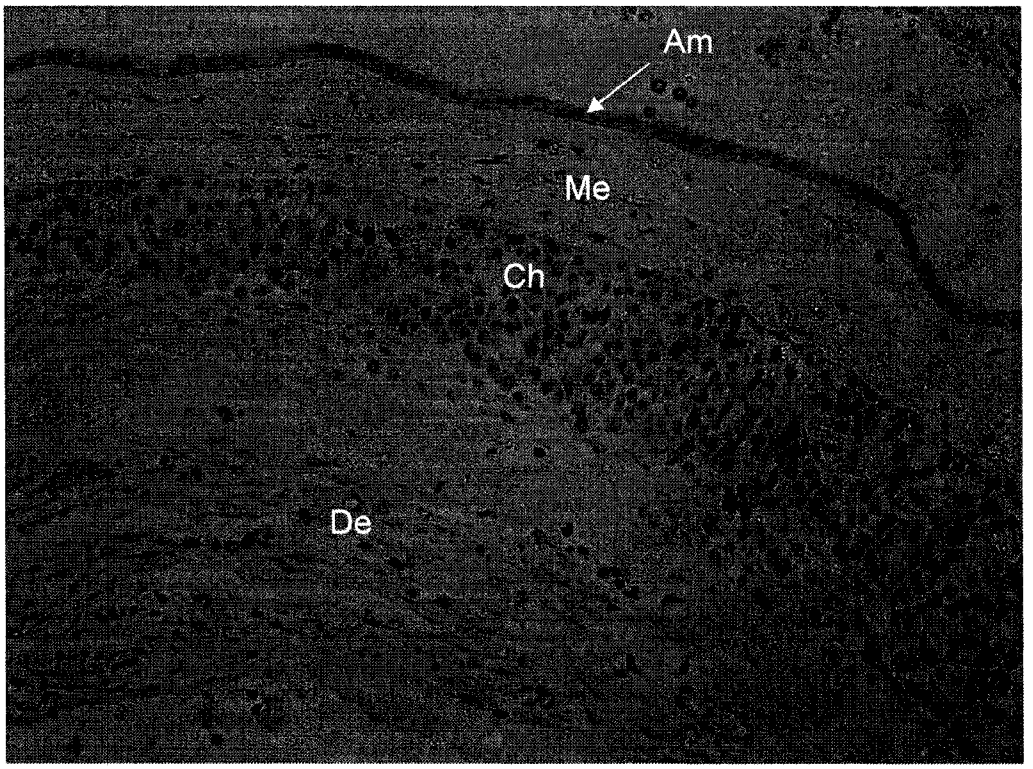

Figure 1.3B: Hematoxylin staining of human fetal membranes demonstrating the arrangement of cells.

The Amnion membrane (Am) is the thin layer of epithelial cells in direct contact with the amniotic fluid. The amnion membrane is separated from the chorion membrane (Ch) by mesenchymal cells (Me). The Decidua (De), which is of maternal origin, is attached to the chorion.

Chapter 2

2 Objectives, Rationale, Hypotheses and Aims

The purpose of the present study was to improve our understanding of the expression of BCRP and its regulation during human pregnancy. Since no invasive studies can be carried out on pregnant women, studies are for the most part limited to human placenta and membranes obtained following labor, whether at term or preterm, termination of early pregnancy and elective C-section at term prior to labor. However, collaborative studies are ongoing at the University of Toronto using the pregnant mouse model as an in vivo system for the study of bcrp1 expression, its regulation and role during pregnancy. Information gathered from these two projects will aid in the elucidation of the physiological and pathophysiological role of BCRP in pregnancy.

2.1 Overall Rationale

BCRP was discovered in multi-drug resistant cancer cell lines only 9 years ago. The protein was found to be highly expressed in many different cancer tissues following its discovery. It was later found to be expressed in many normal tissues, among these the human placenta where mRNA and protein levels of BCRP are very high. Up until the past few years studies on BCRP were mainly in relation to cancer therapy. Within the past three years however efforts have been made to understand the function and

physiological role of BCRP in the human placenta. Studies have been carried out using mice (Jonker *et al.*, 2002;Allen *et al.*, 2002b), placenta derived cell lines (Wang & Baba, 2005;Krishnamurthy & Schuetz, 2005) and, very recently, primary cultures (Evseenko *et al.*, 2007). Even though some information has been gathered about this protein, the role of BCRP in the placenta during pregnancy is not clearly understood.

The **overall objective** of this project was to extend our knowledge of the expression and regulation of BCRP in human placenta and membranes during pregnancy by a) defining the expression pattern and localization of BCRP in the human placenta throughout pregnancy, b) examining BCRP expression in fetal membranes and c) studying the regulation of BCRP expression in the human placenta in vitro.

Our **overall hypothesis** was that BCRP is expressed in the human placenta and fetal membranes throughout gestation. We hypothesized that BCRP expression in human pregnancy is gestational age dependent and is regulated by the chemical and hormonal environment of the tissues expressing BCRP.

2.3 Study 1: Expression of Breast Cancer Resistance Protein (BCRP/ ABCG2) in human placenta throughout gestation and at term before and after labor.

About three years ago Ee et al reported findings of a putative estrogen response element (ERE) at the 5' flanking region of the BCRP gene upon careful examination of the BCRP promoter (Ee *et al.*, 2004). This suggested that estrogen might regulate BCRP

expression. Further studies showed that estrogen increased BCRP mRNA expression in the estrogen receptor positive human breast cancer cell line T47D:A18 and human ovarian cancer cell line PA-1 cells through the estrogen receptor α (ERα) (Ee *et al.*, 2004). However, in another study with MCF-7 cancer cells, BCRP expression was reported to be down regulated by estrogen via the ERα (Imai *et al.*, 2005). Earlier analysis in which functional BCRP was expressed in bacterial *Lactococcus lactis* in the presence of steroids including estradiol reported a stimulation of BCRP function by estradiol, progesterone and testosterone (Janvilisri *et al.*, 2003).

Wang et al have also reported findings that both estrogen and progesterone may regulate BCRP expression. Their study with a placentally derived cell line (BeWo cells) reported a down-regulation of BCRP expression by estrogen and an up-regulation of BCRP expression by progesterone (Wang *et al.*, 2004b). These studies suggest that both estrogen and progesterone may influence the expression and function of BCRP in the placenta.

Since placenta produces increasing amounts of estrogen and progesterone with advancing gestation we **hypothesized** that BCRP expression pattern would change with advancing gestation as a result of the changes in the hormonal environment of the placenta.

The **aim** of the study was to define the expression pattern and localization of BCRP in the human placenta throughout pregnancy.

2.4 Study 2: Expression and Localization of Breast Cancer Resistance Protein (BCRP) in Human Fetal Membranes and Decidua and the Influence of Labor at Term

There is evidence that substances are transported from maternal compartment to the amniotic fluid and vice versa via fetal membranes. Substances that cross the membranes range from chemicals and electrolytes (Lemancewicz *et al.*, 2000) to bacterial, viruses (Rokos *et al.*, 1998) and proteins (Haddow *et al.*, 1979;Mann *et al.*, 2001).

Studies with guinea pig fetal membranes demonstrated a bidirectional transport of sulphated estrone across amnion and chorion tissues (Goldhawk & Hobkirk, 1998). BCRP had been reported to transport estrone and sulfated estrogens in cell lines. Furthermore, studies have indicated the presence of estrogenic agents and dietary xenobiotics including bisphenol A (BPA), daidzein and genistein in fetal serum and amniotic fluid. These compounds are known to be transported exclusively by BCRP (Imai *et al.*, 2004;Enokizono *et al.*, 2007). Therefore we **hypothesized** that BCRP is expressed in fetal membranes and since the fetal membranes play an important role in labor, which involves increased cytokine production and since cytokines have been shown to regulate BCRP expression (Englund *et al.*, 2006), we hypothesized that BCRP expression in fetal membranes would change during labor.

The **aim** of the study was to investigate the expression of BCRP in placental membranes, define its localization and examine the effect of labor on BCRP expression.

2.5 Study 3: Effect of cytokines and hypoxia on BCRP expression in human placenta

Elevation of pro-inflammatory cytokines in gestational tissues and amniotic fluid in response to intrauterine infection has been implicated in preterm birth. There is undeniable evidence that there is a link between increased cytokine production and labor before term (Goepfert *et al.*, 2004;Kataoka *et al.*, 2006;Gonçalves *et al.*, 2002). However, evidence also suggests that cytokines are involved in normal labor at term. For example, under normal conditions, the level of cytokines in gestational tissues and the cervix during labor is higher relative to pre-labor levels (Winkler *et al.*, 1998;Basso *et al.*, 2005;Sennström *et al.*, 2000). In other tissues, cytokines have been shown to alter BCRP expression. Active inflammation of the gut mucosa in patients with ulcerative colitis led to a significant reduction in the levels of BCRP and other multi-drug resistance proteins in this tissue (Englund *et al.*, 2006). We therefore **hypothesized** that BCRP expression in placental trophoblast cells will be decreased by cytokines found during pregnancy, namely, TNFα and IL-1β (Sadowsky *et al.*, 2006).

During the early stages of pregnancy blood flow to the placenta is low. As a result only a reduced amount of oxygen is available to the placenta. This reduced oxygen environment is necessary for normal placental development (Caniggia *et al.*, 2007).

Following extra villous trophoblast invasion of maternal tissues and remodeling of spiral arteries blood flow to the placenta begins to increase increasing the level of oxygen available to the placenta by the latter part of the first trimester and the beginning of the second trimester (Jauniaux *et al.*, 2006). In situations where placental development is affected due to insufficient trophoblast invasion or in some pathological situations the duration of the hypoxic phase may be prolonged and this can have severe consequences on fetal development (Caniggia *et al.*, 2007). A study by Krishnamurthy et al, reported an increase in BCRP mRNA expression in three human derived cell lines and also in SP stem cells under hypoxic conditions (Krishnamurthy *et al.*, 2004). Therefore we **hypothesized** that placental BCRP expression will be increased under hypoxic conditions as has been reported in other cells.

The **aim** of this study was to examine the effect of cytokines and hypoxia on BCRP expression by the human placenta using cultures of human placental trophoblast cells. These culture systems have been well defined over the years and used extensively to examine human placental trophoblast cells in vitro. In these cultures isolated cytotrophoblast differentiate into syncytium.

The results of these three studies are presented as separate articles.

Reference list for chapters 1&2 (page 108-124)

Chapter 3

Expression of Breast Cancer Resistance Protein (BCRP/ ABCG2) in human placenta throughout gestation and at term before and after labor.

Authors: D. Yeboah[1], M. Sun[1] J. Kingdom[3], D. Baczyk[3, 4], S. J. Lye[2, 3, 4], S. G. Matthews[2, 3], and W. Gibb[1].

[1]Department of Obstetrics and Gynecology and Cellular and Molecular Medicine, University of Ottawa, [2]Departments of Physiology and [3]Obstetrics and Gynecology, University of Toronto, and [4]Samuel Lunenfeld, Research Institute, Mt Sinai Hospital, Toronto.

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3.1 Abstract

Breast Cancer Resistance Protein, BCRP, is a multidrug resistance protein that is highly expressed in the human placenta. In cancer tissues, this protein actively extrudes a wide variety of chemically and structurally unrelated chemotherapeutic drugs and other compounds. Studies in mice have shown that in the absence of BCRP activity in the placenta, there is a 2-fold increase in the uptake in BCRP substrates into fetus. This suggests that in the placenta, BCRP extrudes compounds that would otherwise cross the syncytiotrophoblast cells into fetal circulation. The purpose of this study was to examine the expression and localization of BCRP in the human placenta throughout gestation. Tissues from 6-13, 16-19, 24-29, 32-35 and 38-41 weeks of gestation were used. Real time RT-PCR analysis demonstrated that the mRNA levels of BCRP in the placenta do not change significantly as gestation progressed. However, western analysis revealed that the protein levels increased towards the end of gestation. We demonstrated that BCRP is localized to the syncytiotrophoblast of the placenta and in some fetal blood vessels within the placenta. Tissues from the early stages of pregnancy (6-13wks) showed fewer BCRP positive blood vessels than term tissues (38-41wks).

3.2 Introduction

Breast Cancer Resistant Protein, BCRP, is a recently discovered drug transporter that belongs to the ABC super family of proteins (Ross et al 2000). It was first isolated from human multidrug resistant breast cancer cell line, MCF-7/AdrVp, by Doyle et al 1998. Members of the ABC super family actively transport various compounds across intracellular or plasma membranes using energy from ATP hydrolysis (Staud and Pavek 2005, Sarkadi et al 2004). In general xenobiotics and some endogenous compounds such as steroids are transported by these proteins (Dean et al. 2001, Janviliri et al. 2003 and Schmitz et al. 2001). Physiologically, they protect the body from the deleterious effects of toxins and may also regulate the levels of steroid hormones (Dean et al. 2001 and Schmitz et al. 2001). All 49 members of the ABC family, identified by their conserved ATP binding domain and their nucleotide and protein sequence homology, fall into 7 subgroups. BCRP belong to the White or the ABCG subgroup (Dean et al. 2001 and Sarkadi et al. 2004).

BCRP is a half transporter. It has a single nucleotide-binding domain, a six-segment intermembrane domain and a substrate-binding domain. Therefore unlike other ABC transporters such as MDR1 (multi-drug resistance protein 1) which is also highly expressed in the human placenta and functions as a monomer, BCRP must dimerize to become functional (Dean et al. 2001, Schmitz et al. 2001 and Sun et al 2006). BCRP has been shown to transport drugs across the plasma membrane and in a few cases across intracellular membranes (Schmitz et al. 2001). This transporter is able to extrude a wide variety of chemically unrelated compounds that include camptothecins such as SN-38

and topotecan as well as other anti cancer agents such as mitoxantrone, doxorubicin and daunorubicin. BCRP also transports methotrexate and the Hoechst dye 33342 (Allen and Schinkel 2004, Dean et al. 2001, Ross et al. 2000, Sarkadi et al. 2004 and Volk et al. 2002). Studies have shown that naturally occurring single nucleotide polymorphism within the coding region of BCRP affects substrate specificity (Honjo et al. 2002). For instance the wild type BCRP is unable to transport anthracycline, doxorubicin or rhodamine but mutant BCRP in which threonine or glycine replaces arginine at position 482 transport all three substrates (Allen and Schinkel 2004, Sakardi et al. 2004 and Volk et al. 2002).

Since its discovery in breast cancer cells, BCRP has been found in drug resistant ovary, colon and gastric cancer cells (Young et al. 2003). BCRP is also expressed in normal cells. These include the epithelia of the intestines and colon, duct and lobules of the breast, the epithelial cells of veins and capillaries of these tissues and the syncytiotrophoblast of the placenta (Maliepaard et al. 2001 and Young et al. 2003).

Little is known of the physiological function and regulation of BCRP in normal tissues. Most of the work done on this protein to date is in the area of tumorigenicity and therapeutics. In mice, inhibition of Bcrp1, the mouse analogue of BCRP, by GF120918 leads to an increase in the uptake topotecan in the fetus (Jonker et al. 2002). This suggests that BCRP in the placenta prevents the accumulation of drugs in the fetus and protects it from the harmful effects of these drugs.

Only a few studies mention the presence of BCRP in the human placenta. For the most part, these studies present limited data on BCRP expression in term tissue, using immunohistochemical approaches to localize BCRP to the syncytiotrophoblast and western analysis to estimate BCRP levels (Diestra et al. 2002, Litman et al. 2002 Maliepaard et al. 2001). A recent study has described BCRP expression in the human placenta at different periods of gestation (Mathias et al. 2005) but this study was limited to examining isolated syncytial microvillous plasma membrane and a very limited number of tissues were studied. In the present study, we have quantified the expression of BCRP mRNA and protein in the whole placenta at different gestational ages, from very early in gestation (6-13wks) up to term and examined the effect of labor on BCRP expression at term. Since the active form of BCRP is believed to be multimeric (Kage et al. 2002; Litman et al 2002) we also examined its state at early and late gestation using western analysis under non-reducing conditions. In addition, its localization from early gestation (6-13wks) to term before and after labor was defined using immunohistochemistry.

3.3 Materials and Methods

Tissue preparation

Placentae tissues were obtained after term delivery or cesarean section (38-41wks), preterm delivery and after termination of early stage pregnancy from the Ottawa General Hospital and Mount Sinai Hospital, Toronto, with ethical committee approval at both institutions. A portion of the tissues were fast frozen in dry-ice chilled isopentane and stored at –80°C for Western analysis and RT-PCR. The rest of the tissues were fixed in 4% Bouin's and paraffin embedded for immunohistochemistry.

Western blotting

Tissues 0.3g to 0.4g were homogenized in 1ml of RIPA lysis buffer containing; 1% Triton X-100, 1% SDS (Sigma MO, USA), 0.15M NaCl (EM Science NJ, USA), 15.4mM Tris-HCl (BioRad CA, USA), 0.5% deoxycholic acid (Sigma MO, USA) and 100μM sodium orthovanadate supplemented with protease inhibitors ("Complete"; Roche Diagnostics, Germany). Protein concentration was measured with BioRad protein assay (BioRad, Richmond, CA, USA). Duplicates of samples containing 5μg and 10μg of protein per sample were diluted in an equal volume of Laemmli sample buffer (BioRad) and separated on 10% polyacrylamide gel. This was done to ensure that both BCRP and Gβ protein on the blots were in their linear range of absorption. Proteins were then transferred onto pure nitrocellulose blotting membrane (VWR international) by electrobloting at 100V for 1hr. Non-specific binding was blocked overnight with 5% non-fat dry milk (BioRad) in Phosphate Buffered Saline, PBS, containing 2.67mM KCl

(Aldrich WS, USA), 1.48mM KH_2PO_4 (Aldrich WS, USA), 13.7mM NaCl and 8mM Na_2HPO_4 (BDH Canada) and 0.05% Tween (BioRad). Membrane was incubated overnight at 4°C with a mouse monoclonal anti human BCRP antibody, BXP-21, obtained from Calbiochem, (1:250) or an equal concentration of a non-immune mouse IgG for control. This was followed by incubation with HRP-conjugated sheep antimouse IgG from Amerhsam, England (1: 1000) for 1hr. Proteins were visualized by chemiluminescence detection with the ECL system (Amerhsam) and Kodak BioMax MR-1 film (Amersham, England). For experiments under non-reducing conditions β-mercaptoethanol was omitted from the sample buffer. These samples were run on 7.5% polyacrylamide gels. Bands were quantified by densitometry with Gβ (T-20; Santa Cruz Biotechnology CA) as an internal loading control.

Immunohistochemistry

Bouin's fixed (saturated picric acid, 100% formaldehyde and glacial acetic acid in the ratio 15: 5:1) paraffin embedded tissues blocks were cut into 10μm sections and fixed onto Superfrost Plus slides (Fisher Scientific, Fair Lawn, NJ) by baking at 43°C overnight. Tissues were deparaffinized in xylene and re-hydrated in a graded series of ethanol dilutions. After rinsing in water and PBS twice sections were microwaved at high power for a 1min then at 30% power for 10min in 10mM Citric acid (BioRad) pH 6.0 to unmask BCRP sites. Slides were left to cool for 15min and were washed in water and PBS for 5min each. Endogenous peroxidase activity in the tissues was blocked with incubation in 0.3% (v/v) H_2O_2 (BDH England) in methanol (EMD NJ, USA) for 30min. Slides were washed for 10min in PBS twice and incubated in 1.5% of normal horse

serum (Vector Laboratories, CA) in PBS for 20min to block non-specific binding sites. Sections were then incubated in BXP-21 (1:100) or an equivalent amount of non-immune mouse IgG overnight at 4°C. For vimentin labeling a 1:5000 dilution of the antibody was used (Dako Denmark). This was incubated for an hour. Following the incubation, slides were washed in PBS for 5min twice and incubated with a rabbit anti mouse biotinylated antibody for 1hr. After 2 (5min) washes and then treated with an avidin-biotin complex for 1hr. All reagents were from the Vectastain Elite ABC kit (Vector Laboratories, CA). Proteins were visualized using DAB peroxidase substrate (Sigma Chemical Co., MO). Slides were counterstained with haemotoxylin (EMD), dehydrated in ethanol (EM Science), cleared in xylene compound (EM Science) and mounted with permount (Fisher Scientific, NJ).

Real time RT-PCR

Total RNA from frozen tissues was extracted using the Qiagen RNeasy Mini kit and treated with TURBO DNA free set (Ambion, Tx) to remove DNA. The real time RT-PCR was done in one step using the TaqMan One-Step RT-PCR Master Mix Reagents Kit and the ABI PRISM 7000 apparatus (Applied Biosystems, CA) according to manufacturer's instruction. Primers and probe sequences for BCRP were designed with the Primer Express 2.0 software (Applied Biosystems, CA) and checked with BLAST to minimize cross reactivity with other sequences. The following sequences were used: BCRP forward 5'-GGCTTCAGTACTTCAGCATTCCA-3', BCRP reverse 5'-AGTCCTGGGCAGAAGTTTTGTC-3' and hybridization probe for BCRP 5'-FAM-ATGGATTTACGGCTTTGCAGCATAATGAATTTTT-TAMRA-3'supplied by Applied

Biosystems. GAPDH was used as the internal loading control. GAPDH control reagents including primers and probe were designed and supplied by Applied Biosystems (product number 402869). The one-step RT-PCR was done in a 20µl reaction containing 1x TaqMan Master Mix, 1x MultiScribe and RNase inhibitor Mix, 100ng of RNA, 0.125µM of each primer and 0.2µM of probe for BCRP. For GAPDH 0.25µM of each primer and 0.125µM of probe were used. Reverse transcription was performed at 48°C for 30min and stopped at 95°C for 10min followed by 40 cycles of amplification at 95°C for 15 sec and 60°C for 1 min. A standard curve was constructed with serial dilutions of RNA for analysis of the copy number of the target and the control gene in each sample. The results were normalized using the ratio of the target DNA concentration to that of the control DNA (GAPDH). All experiments were run in triplicates.

Statistical analysis

Statistical differences were determined using ANOVA (single factor) and Tukey's t-test with significance set at $p \leq 0.05$.

3.4 Results

Quantitative real-time PCR was performed with placental tissues from 6-13wks of gestation, 16-19 wks, 24-29wks, 32-35 wks of gestation and term (38-41 wks) before and after labor. BCRP mRNA was highly expressed by 6-13 weeks of gestation, and levels did not change significantly as gestation progressed. Nor was there any significant change in expression during labor at term (Figure 3.1). Sequencing of the PCR product confirmed that a portion of the BCRP message was detected and amplified.

Western analysis was performed to assess the protein level of BCRP at these gestational ages. BXP-21, a monoclonal antibody raised against human BCRP, was selected because it has been shown to work well for both western analysis and immunohistochemistry (Diestra et al. 2002 and Maliepaard et al. 2001). Western analysis demonstrated BCRP expression in the human placenta throughout gestation. Under reducing conditions the molecular weight of the protein was approximately 72kDa (Figure 3.2A). The level of the 72kDa protein was quantified by densitometry at the various gestational ages stated above. The level of BCRP relative to Gβ protein expressed at term was significantly higher ($p=0.01$) than that expressed from 6-13wks of pregnancy (Figure 3.2B). Also, BCRP protein levels in all other preterm tissues were significantly ($p<0.05$) lower than those in term labor tissues. BCRP protein levels at term did not differ significantly before or after labor. However, a higher variation was observed in C-section tissues. Even though there was a slight increase in the amount in BCRP protein at the period between 24-35wks, this

Figure 3.1

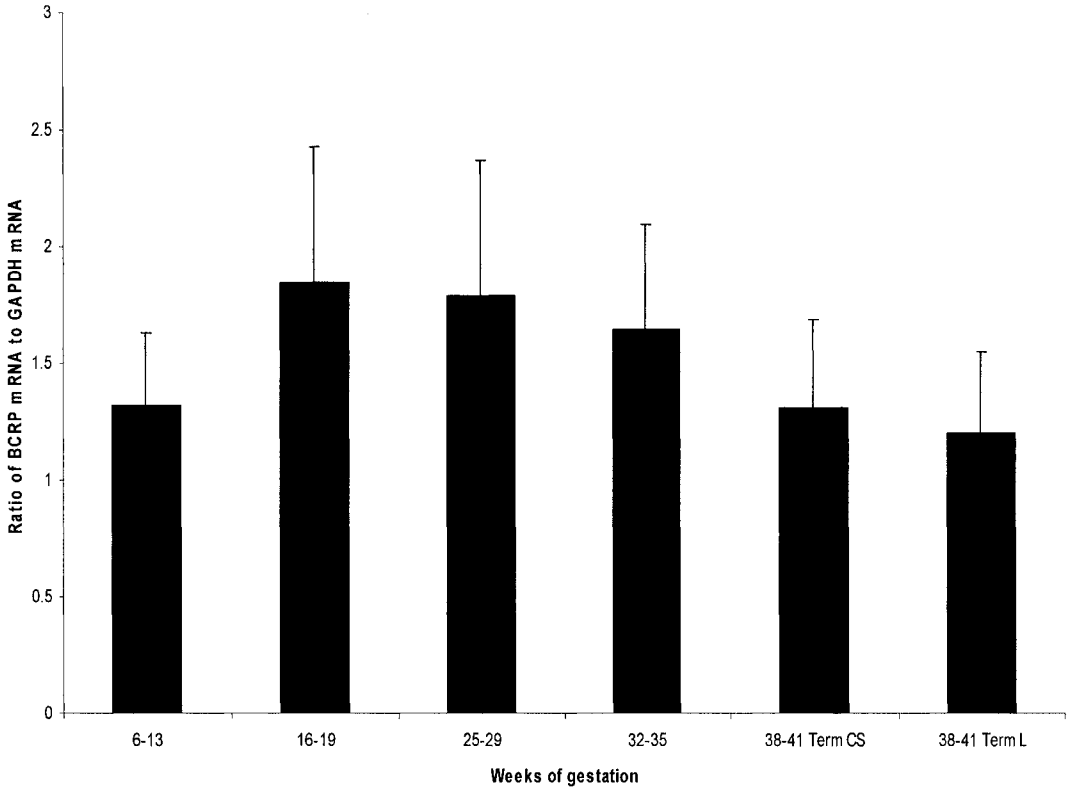


Figure 3.1: Quantitative real time RT-PCR of BCRP mRNA in human placentae

Comparison of the expression of BCRP mRNA in human placentae of different gestational ages. Mean ±SEM of the ratio of BCRP mRNA to GAPDH mRNA in tissues from 6-13wks; (n=6), 16-19wks; (n=6), 24-29wks;(n=5), 32-35wks; (n=6) and term (38-41wks n=8) before and after labor. P>0.05 between all groups.

did not reach statistical significance when compared to that of tissues obtained from 6-13wks of gestation.

In order to examine whether oligomers of BCRP were present at each gestational age, electrophoresis was carried out under non-reducing conditions. A single band of approximately 200kDa was observed (Figure 3.2C). This molecular weight was also observed when the samples were treated with the chemical cross linker, DSP (data not shown).

BCRP protein was also localized in the tissues of different gestational ages using immunohistochemistry. BCRP was present in the syncytiotrophoblast and the endothelial cells of some fetal blood vessels of the placenta. This localization was observed throughout gestation (Figure 3.3A). As it is difficult to differentiate arteries or veins in the placenta villi under the microscope, we examined BCRP expression in the umbilical cord as the two arteries and one vein are clearly defined. However, in the umbilical cord samples neither the arteries nor vein were found to express BCRP (data not shown). We quantified the number of positively stained fetal blood vessels in tissues from early gestation and term and the number of fetal capillaries that positively stained for BCRP was higher in term tissues than in tissues obtained from 6-13wks gestation (Figure 3.3B and Table 1).

Figure 3.2

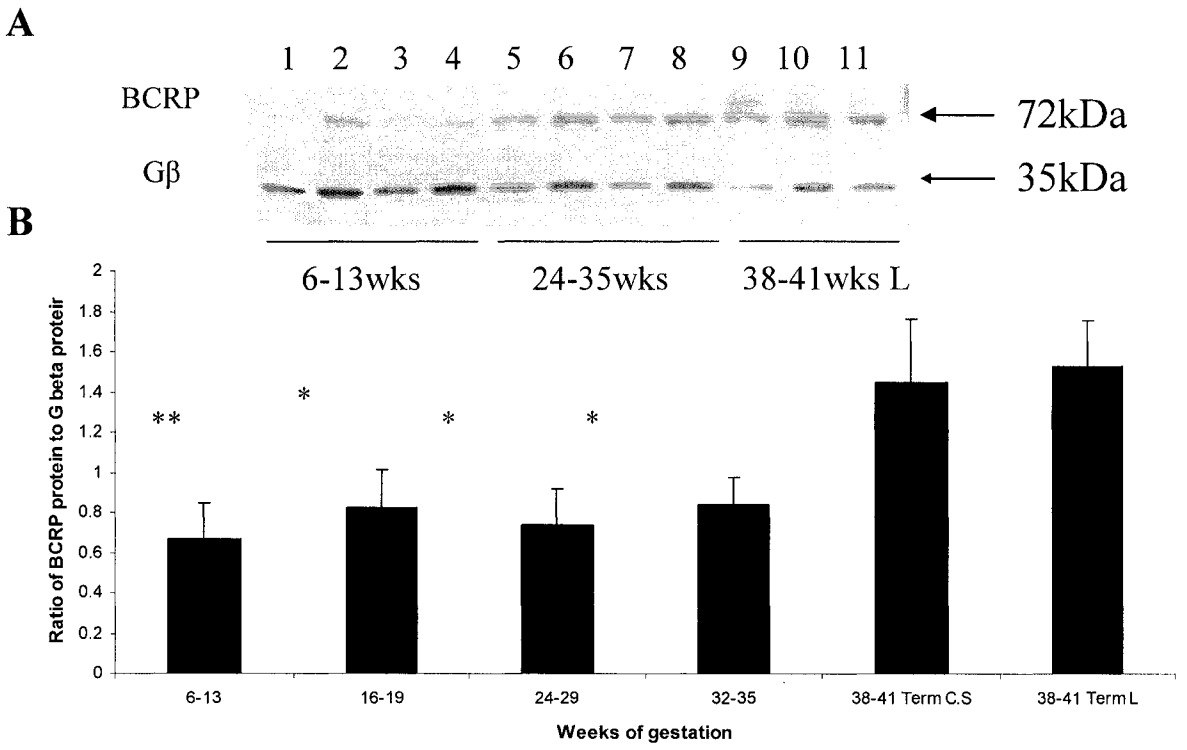
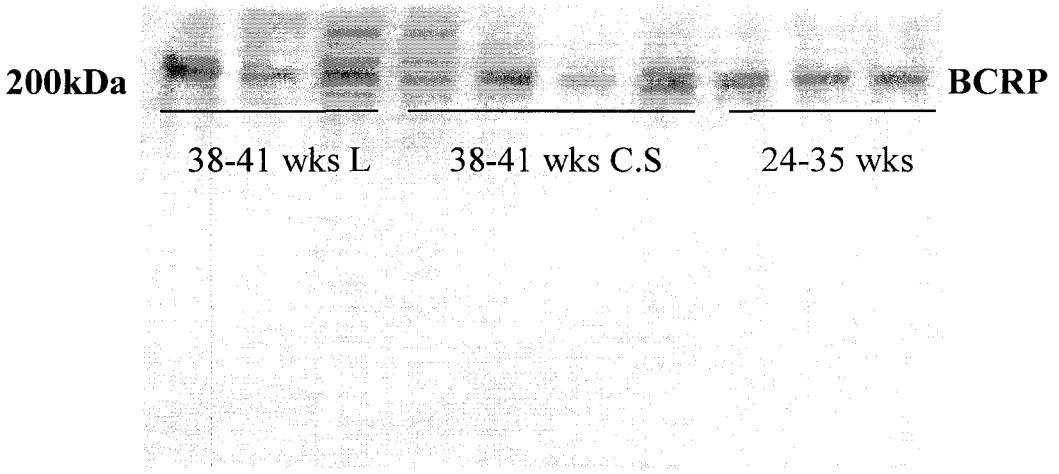


Figure 3.2: Western analyses of BCRP at different periods of gestation.

A: Typical Western blot with BXP-21antibody shows the presence of BCRP, 72kDa protein, throughout gestation. Gβ was used as loading control. Protein samples were loaded in duplicates of 5μg and 10μg per sample to ensure that both BCRP and the loading control were within the linear range of detection. Proteins from gestational ages 16-19wks and 38-41wks C. section (not on this blot) were also run and quantified.

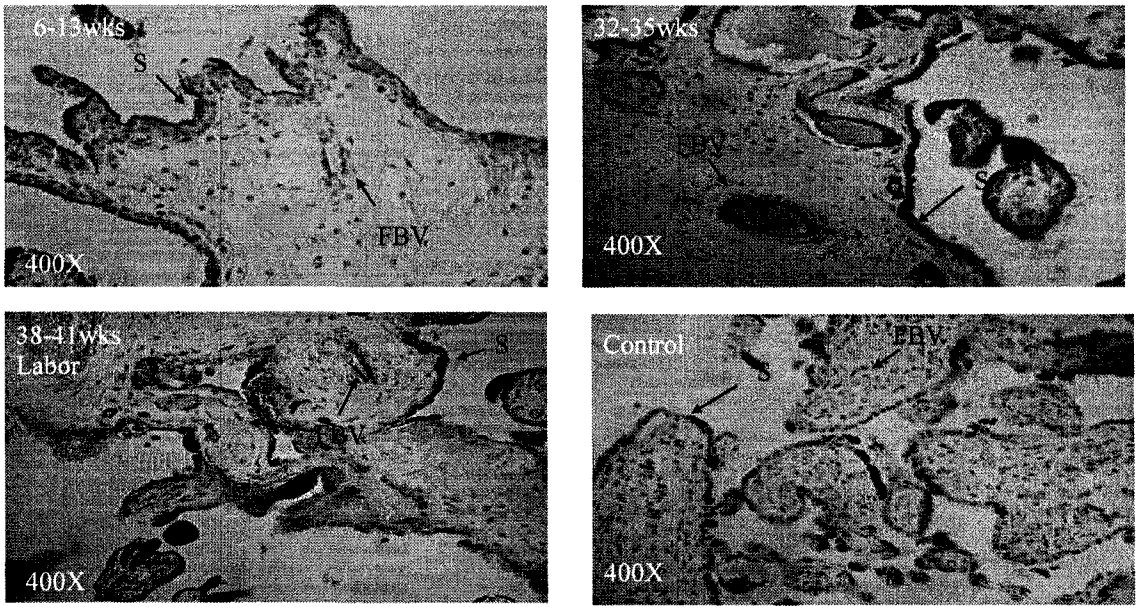
B: Quantification of BCRP in human placentae throughout gestation by densitometry. The 72kDa BCRP protein was quantified at 6-13, 16-19, 24-29, 32-35 and 38-41 weeks of gestation. This analysis showed that BCRP protein level increased with advancing gestation. **P=0.01 between term labor and 6-13wks and *P≤0.05 between term labor tissues and all other preterm tissues. The number of tissues in each group is 8.



C: Under non-reducing conditions the 72 kDa protein was absent from samples of all gestational ages. However, an approximately 200kDa protein was present in all samples. Proteins from gestational ages 16-19wks and 38-41wks (data not shown) were also run.

Figure 3.3

A



B

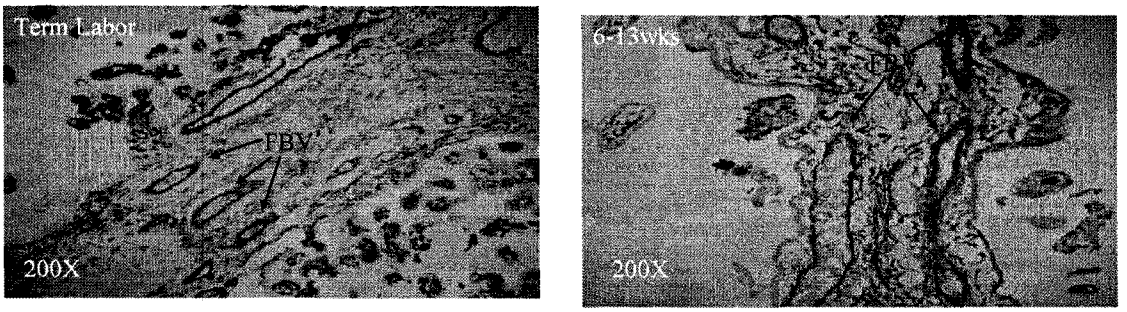


Figure 3.3: Immunohistochemical localization of BCRP in human placenta throughout gestation.

A: BXP-21, monoclonal anti-BCRP antibody was used in dilution 1:100. BCRP is localized to the syncytiotrophoblast (S) and to some fetal blood vessels (fbv) of the placenta. Pre-immune mouse IgG was used for control.

B: Fetal blood vessels (fbv) labeling with vimentin at 6-13 weeks gestation and term.

Table I: Analysis of BCRP expression in fetal blood vessel.

Gestational ages	Analysis of BCRP positive fetal blood vessel of early gestation and Labor tissues. 6-13 wks	Term (38-41wks) labor
Total number of BCRP positive FBV per 10 randomly selected villi	6	51
Total number of vimentin positive FBV per 10 randomly selected placenta villi	40	77
Percentage	15%	66%

BCRP positive fetal blood vessels are averages from 10 randomly selected villi in 4 term and 4 early gestation tissues. This was compared to the average number of fetal blood vessels present in 10 randomly selected villi of these tissues as determined by vimentin staining see Figure 3B. The expression of BCRP in fetal blood vessels increased as gestation progressed.

3.5 Discussion

This study demonstrates that BCRP is expressed in the human placenta throughout gestation. Although the level of BCRP mRNA in the placenta did not change significantly as gestation progressed or during labor at term, BCRP protein levels increased significantly at term. BCRP was found to be localized not only in the syncytiotrophoblast but also in the endothelial cells of some fetal blood vessels at all stages of gestation. The number of fetal blood vessels expressing BCRP at term was found to be greater than that found early in gestation. Finally, we demonstrate that, BCRP is present as a multimeric complex, either in association with itself and/or with other proteins at all gestational ages.

One other study has examined the level of BCRP in the human placenta at different gestational ages (Mathias et al. 2005). This study examined only a limited number of tissues, and used isolated microvillous membrane preparations. It did not examine tissues between 17 weeks of gestation and term. Substantial variation in both mRNA and protein levels of BCRP, at different gestational ages was found and it was concluded that BCRP mRNA and protein levels remained unchanged throughout gestation. An in house ELISA was developed for protein quantification in this study. Our data indicate that, while placental BCRP mRNA levels do not change significantly throughout gestation, BCRP protein levels are increased significantly towards the end of gestation. In contrast to the study by Mathias et al 2005 we used whole placenta tissue and since we observed that the proportion of BCRP-positive fetal capillaries increased with advancing gestation this may explain the increase in BCRP protein levels that we found to occur. Future studies using

laser capture micro-dissection to isolate specific cell types in the placenta for analysis of mRNA and protein levels of BCRP may allow one to directly assess this possibility. At the present time we cannot exclude the possibility that the increase is due to an increase in the syncytiotrophoblast. Indeed, recently Evseenko et al suggested that BCRP expression in trophoblast cells increased with differentiation and term placentas have more differentiated cytotrophoblast than preterm tissues. The discrepancy between message and protein levels of BCRP that we found is most likely due to post transcriptional regulation of protein synthesis.

Not all fetal blood vessels in the placenta expressed BCRP. It has been shown that BCRP expression is stimulated under hypoxic conditions in stem cells (Krishnamurthy et al. 2004). Since in the placenta the fetal arteries carry deoxygenated blood coming from the fetus, we speculate that the low oxygen environment may stimulate BCRP expression. In other tissues such as liver, small intestines and colon where veins transport deoxygenated blood; BCRP has been detected in endothelial cells in venous capillaries (Maliepaard et al. 2001). As it is difficult to differentiate between arteries and veins in the placenta, we examined the umbilical cord tissue in which the arteries (two arteries) and a vein are easily identified. Unfortunately, in the umbilical cord neither the vein nor arteries expressed BCRP. This indicates that BCRP expression in the epithelial cells of the blood vessels may be regulated by the local action of factors derived from the placenta itself.

It has been shown by our laboratory and others that the expression of Mdr-1/P-gp, another major multi drug resistant protein in the placenta, drops significantly as gestation

progress (Mathias et al. 2005 and Sun et al. 2005). It is possible that the increase in BCRP protein expression may be a compensatory mechanism to counterbalance the decrease in Mdr-1/P-gp expression. Indeed, in the Mdr-1/P-gp knockout mouse placenta, we found that BCRP expression is higher than in the wild-type controls (unpublished observation). This raises the possibility that factors that activate the expression of either one of these drug transporters may repress the activity and/or the expression of the other.

Term tissues were examined both prior to and following labor in order to determine whether labor had any influence on BCRP expression. Currently, regulation of BCRP expression in the human placenta is not clearly understood. It has been reported that hypoxia may regulate BCRP expression (Krishnamurthy et al 2004). Tissues obtained following labor are potentially more hypoxic than term C-section tissues. Also, estradiol and progesterone which are involved in labor (Madsen et al. 2004) have been implicated in BCRP regulation (Wang et al. 2005).

The 5' flanking region of the BCRP gene suggest the presence of an estrogen response element (ERE) (Ee et al. 2004). However, there are conflicting reports on the effect of estrogen on BCRP expression in mammalian cells. In one study, estrogen was found to up regulate BCRP mRNA expression in T47D:A1 and PA-1 cells through the estrogen receptor α (ERα) (Ee et al. 2004). In another study with MCF-7 cancer cells, BCRP expression was down regulated by estrogen via the ERα (Imai et al. 2005). But when functional BCRP was expressed in bacterial, *lactococcucs lactis,* BCRP activity was found to be stimulated by estradiol, progesterone and testosterone (Janviliri et al. 2003).

These studies suggest that estrogen may play a regulatory role in BCRP expression. In a recent study by Wang et al. 2005, increasing concentrations of estrogen reduced BCRP mRNA and protein levels while increasing concentrations of progesterone increased BCRP mRNA and protein levels in human placental BeWo cells. In the presence of both hormones however, BCRP expression (both mRNA and protein) was found to increase. This may be particularly important in human pregnancy as both estrogen and progesterone levels increase with advancing gestation but further studies will be necessary to determine if these factors are important in regulating BCRP expression in the human placenta.

BCRP is considered to be a plasma membrane protein that functions in complex with itself or with other proteins (Diestra et al. 2002, Kage et al. 2002 and Litman et al. 2002). These studies reported a protein of a molecular weight greater than 72kDa when BCRP is detected on SDS gels, under non-reducing conditions. It has been suggested that BCRP most likely functions as a homodimer based on immunoprecipitation results showing a band of ~140kDa and also experiments showing loss of BCRP function when co-expressed with a nonfunctional mutant (Kage et al. 2002). It is also possible that BCRP functions in a complex with other proteins, as under non-reducing conditions and in the presence of a cross-linker a molecular weight higher than 140 kDa is observed (Litman et al. 2002). This would be consistent with our present findings. These high molecular weight protein complexes were not quantified. Findings from this experiment imply that the BCRP proteins found at the very early stages of pregnancy (6-13wks) as well as those of the other gestational ages have the potential to be active since the 72kDa protein was

missing in all samples under non-reducing conditions. Also, no one has studied this form of the protein in human placenta tissue. In other cell types a 140kDa protein is observed under non-reducing conditions (Kage et al. 2002). Our finding of a higher molecular weight proteins suggest that BCRP may function as a multimer instead of a dimer in the human placenta.

BCRP is expressed in the human placenta and, like other placental drug transporters; it may prevent harmful toxins from reaching the fetus by actively transporting these toxins across cell membranes (Allen and Schinkel 2004). In addition it may be involved in folate and steroid transport; factors which are particularly important during human pregnancy (Ifergan et al. 2004 and Schmitz et al. 2001). The present study has defined the expression and localization of BCRP in human placenta throughout gestation. This information will form the foundation for future studies focused towards defining the physiological role of BCRP in pregnancy together with the mechanisms involved in its regulation in normal and compromised pregnancies.

Reference list for chapter 3 (page 125-127)

Chapter 4

Expression and Localization of Breast Cancer Resistance Protein (BCRP) in Human Fetal Membranes and Decidua and the Influence of Labor at Term

Authors: D. Yeboah[1], GM Kalabis[3], M Sun[1], R C Ou[1], SG Matthews[3] and W Gibb[1,2]

[1]Department of Obstetrics and Gynecology and Cellular and Molecular Medicine, University of Ottawa, [2]Ottawa Health Research Institute, and [3]Departments of Physiology, Medicine and Obstetrics & Gynecology, University of Toronto

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4.1 Abstract

Breast cancer resistance protein, BCRP, is a multidrug resistant ABC transport protein, ABCG-2. It extrudes a wide rage of substrates including many chemotherapy drugs, steroids and folate. It is present in many cancers as well as normal tissues, in particular, barrier tissues such as the blood brain barrier, the intestine, blood vessels and the human placenta. The human fetal membranes (amnion and chorion leave) provide the barrier between the maternal uterine environment and the fetus. In this study we defined the expression and localization of BCRP mRNA and protein in human fetal membranes (amnion and chorion) and attached decidua obtained prior to and following labor at term. BCRP protein and mRNA was expressed in all tissues examined and the levels of expression were not altered by labor. BCRP was localized to the amnion epithelial cells, chorion trophoblast cells and decidua stromal cells as well as the endothelial cells of maternal blood vessels in decidua, but was absent from the mesenchymal cells. In the amnion epithelium the protein was localized to the apical surface, cytoplasm and membrane between cells. In the chorion trophoblast and decidua stromal cells the protein was localized to the plasma membrane. In the chorion trophoblast the protein was also highly expressed in the nucleus. The level of BCRP protein in the membranes was comparable to the placenta. These high levels raise the possibility that this transporter may play an important role in the physiological function of the tissues.

4.2 Introduction

Breast Cancer Resistance Protein, BCRP, was first isolated from the human multidrug resistant breast cancer cell line, MCF-7/AdrVp, by Doyle et al (1998)[1]. BCRP is a member of the ABC super family of proteins, which are known to actively transport various compounds across intracellular or plasma membranes[2, 3]. All ABC proteins have a conserved ATP binding domain and are classified into 7 subgroups. BCRP belongs to the 'White' or the ABCG subgroup and is also known as ABCG2[3,4,5]. BCRP is a half transporter; it has a single nucleotide-binding domain, a six-segment transmembrane domain and therefore must form a complex to become functional[3, 5].

BCRP is able to efflux a wide range of compounds that differ chemically and structurally. These include many chemotherapy drugs such as mitoxantrone, doxorubicin and camptothecins (SN-38 and topotecan)[3,6] as well as other compounds including BODIPY-prazosin and the Hoechst dye 33342 [7, 8]. BCRP has also been shown to transport certain steroids, such as DHEAS[9] and estradiol[10], and is also involved in cellular folate homeostasis as it transports folate and the anti-folate methotrexate [11]. Evidence suggests that steroids and folate may regulate BCRP expression[9,10,11].

Following its discovery in breast cancer and other tumor tissues cells[12], BCRP has been shown to be in normal cells. Non-cancer cells expressing BCRP include the epithelia of the intestines and colon, the duct and lobules of the breast, the endothelial cells of veins and capillaries in these tissues and the syncytiotrophoblast of the placenta[12, 13]. Studies

with pregnant mice have shown that inhibition of Bcrp1, the mouse analogue of the human BCRP, by GF120918, led to an increase in the uptake topotecan in the fetuses[14]. This suggests that BCRP in the placenta may prevent the accumulation of certain drugs, thus protecting the fetus from their potentially harmful effects.

BCRP is expressed at high levels in the human placenta[13,15,16] and its expression throughout gestation has been described[17,18]. In addition, regulation of BCRP in human placental cultures and in cell lines derived from placenta has been examined in a number of studies[19-22]. Among the factors that alter BCRP expression in primary cultures of human placental trophoblast, cytokines were found to decrease BCRP expression and estrogens increase BCRP expression [23].

The fetal membranes (amnion and chorion laeve), which extend from the placenta, are an important barrier between the fetus and the mother. These membranes not only regulate access of substances to and from the amniotic fluid and hence the fetus, but are also intimately involved in the onset of labor[24]. Very recently Aye at al described the presence of multidrug resistance transporters, including BCRP, in human fetal membranes[25]. These studies were principally confined to the amnion though the presence of the protein in the chorion and decidua was mentioned. The purpose of the present study was to define, the expression of BCRP in fetal membranes (amnion and chorion leave) and attached decidua. Since cytokines production in the membranes increase during labor[26-28] and cytokines have been shown to regulate BCRP expression in other tissues and cell types[23, 29], we hypothesized that labor would alter BCRP expression in these tissues. Using

tissues obtained before and after labor at term we have quantified BCRP mRNA and protein using Real–time PCR and Western analysis, respectively. We have also carried out detailed immunohistochemical analysis to examine the localization of BCRP in the amnion, chorion leave and decidua.

4.3 Materials and Methods

Tissue preparation

Fetal membrane and placental tissues were obtained after term vaginal delivery or cesarean section (38-41wks), from the Ottawa General Hospital with approval of the ethics committee. Amnion was separated from chorion/decidua and a portion of the tissue was fast frozen in dry-ice chilled isopentane and stored at –80°C for Western analysis and RT-PCR. Tissues for immunohistochemistry were rolled and fixed in 4% Bouin's solution and embedded in paraffin[30].

Western blotting

Tissues 0.3g to 0.4g were homogenized in 1ml of RIPA lysis buffer containing; 1% Triton X-100, 1% SDS (Sigma MO, USA), 0.15M NaCl (EM Science NJ, USA), 15.4mM Tris-HCl (BioRad CA, USA), 0.5% deoxycholic acid (Sigma MO, USA) and 100μM sodium orthovanadate supplemented with protease inhibitors ("Complete"; Roche Diagnostics, Germany). Protein concentration was measured with BioRad protein assay (BioRad, Richmond, CA, USA). Duplicates of samples containing 5μg of protein were diluted in an equal volume of Laemmli sample buffer (BioRad) and separated on 4-15% polyacrylamide gels (BioRad). Proteins were then transferred onto pure nitrocellulose blotting membrane (VWR international) by electrobloting at 100V for 1hr. Non-specific binding was blocked overnight with 5% non-fat dry milk (BioRad) in Phosphate Buffered Saline, PBS, containing 2.67mM KCl (Aldrich WS, USA), 1.48mM KH_2PO_4 (Aldrich WS, USA), 13.7mM NaCl and 8mM Na_2HPO_4 (BDH Canada) and 0.05% Tween (BioRad). Membrane was incubated overnight at 4°C with a mouse

monoclonal anti human BCRP antibody, BXP-21, obtained from Calbiochem, (1:250), or with an equal concentration of a non-immune mouse IgG for controls. This was followed by incubation with HRP-conjugated sheep anti mouse IgG (1: 1000, 1hr; Amersham, UK) Proteins were visualized by chemiluminescence detection with the ECL system (Amersham) and Kodak BioMax MR-1 film (Amersham, England). Bands were quantified by densitometry with Gβ (T-20; Santa Cruz Biotechnology CA) as an internal loading control (1:5000)[18,30,31]. Comparison of BCRP protein in the fetal membranes and placenta were carried out at the same time using the same batch of antibody.

Immunohistochemistry

Bouin's-fixed (saturated picric acid, 100% formaldehyde and glacial acetic acid in the ratio 15: 5:1) paraffin-embedded tissues blocks were cut into 10μm sections and fixed onto Superfrost Plus slides (Fisher Scientific, Fair Lawn, NJ) by baking at 43°C overnight. Immunohistochemical analysis was carried out as previously indicated[18,30]. Briefly, tissues were deparafinized in xylene and re-hydrated in a graded series of ethanol dilutions. After rinsing in water and PBS twice, sections were placed in a microwave at high power for a 1min then at 30% power for 10min in 10mM Citric acid (BioRad) pH 6.0 to unmask antibody-binding sites. Slides were left to cool for 15min and were washed in water and PBS for 5min each. Endogenous peroxidase activity in the tissues was blocked by incubation in 0.3% (v/v) H_2O_2 (BDH England) in methanol (EMD NJ, USA) for 30min. Slides were washed for 10min with PBS (2X) and incubated in 1.5% horse serum (Vector Laboratories, CA) in PBS for 20min to block non-specific binding sites. Sections were then incubated in BXP-21 (1:100 v/v) or an equivalent amount of non-

immune mouse IgG for 1h at room temperature. After washing in PBS for 5min (2X), slides were incubated with a rabbit anti mouse biotinylated antibody for 30min then washed and incubated with an avidin-biotin complex, all from the Vectastain Elite ABC kit (Vector Laboratories, CA) for 30min. Proteins were visualized using DAB peroxidase substrate (Sigma Chemical Co., MO). Slides were counterstained with haemotoxylin (EMD), dehydrated in ethanol (EM Science), cleared in xylene compound (EM Science) and mounted with permount (Fisher Scientific, NJ).

Real time RT-PCR

Total RNA was extracted from 30mg of frozen tissue using the Qiagen RNeasy Mini kit and DNase treated using the RNase-free DNase set (Qiagen, ON Canada). Real time RT-PCR was done in one step using the TaqMan One-Step RT-PCR Master Mix Reagents Kit and the ABI PRISM 7000 apparatus (Applied Biosystems, CA) according to manufacturer's instruction. Primers and probe sequences for BCRP were designed with the Primer Express 2.0 software (Applied Biosystems, CA) and checked with BLAST to minimize cross reactivity with other sequences. The following sequences were used: BCRP forward 5'-GGCTTCAGTACTTCAGCATTCCA-3', BCRP reverse 5'-AGTCCTGGGCAGAAGTTTTGTC-3' and hybridization probe for BCRP 5'-FAM-ATGGATTTACGGCTTTGCAGCATAATGAATTTTT-TAMRA-3'supplied by Applied Biosystems[18]. The PCR product was sequenced to confirm that the expected sequence was amplified. GAPDH was used as the internal control[18,30]. GAPDH control reagents including primers and probe were designed and supplied by Applied Biosystems (product number 402869). The one-step RT-PCR was done in a 20µl reaction containing 1x

TaqMan Master Mix, 1x MultiScribe and RNase inhibitor Mix, 100ng of RNA, 0.125μM of each primer and 0.2μM of probe for BCRP. For GAPDH 0.25μM of each primer and 0.125μM of probe were used. Reverse transcription was performed at 48°C for 30min and stopped at 95°C for 10min followed by 40 cycles of amplification at 95°C for 15 sec and 60°C for 1 min. A standard curve was constructed with serial dilutions of RNA for analysis of the copy number of the target and the control gene in each sample. The results were normalized using the ratio of the target DNA concentration to that of the control DNA (GAPDH). In the series of experiments to compare mRNA levels in the membranes these tissues were analyzed at the same time. All experiments were run in triplicate for each tissue examined.

Statistical analysis

Statistical differences were determined using ANOVA (single factor) with significance set at $p \leq 0.05$.

4.4 Results

Real-time PCR was used to quantify BCRP mRNA in amnion and chorion/decidua obtained before and after labor at term (38-41 weeks gestation). In all tissues BCRP mRNA was expressed but there was no significant difference in the levels expressed in tissues obtained prior to and following labor (Figure 4.1A and B). Although in the amnion BCRP message levels were higher in tissues obtained following labor (Figure 4.1A) this did not reach statistical significance.

Western analysis was performed to assess the protein levels of BCRP in amnion and chorion/decidua tissues. The BXP-21, a monoclonal antibody raised against human BCRP was selected, as it was suitable for both Western analysis and immunohistochemistry [11,14]. BCRP protein was present in all tissues examined. Under reducing conditions the molecular weight of the protein was approximately 72kDa (Figure 4.2A & C). A slightly higher molecular weight protein was also detected (Figure 4.2C). The levels of the 72kDa protein were quantified by densitometry. The levels of BCRP were the same in amnion and chorion/decidua obtained before and after labor (Figure 4.2B and D). Since placenta contains high levels of BCRP protein we compared the levels in the membranes with this tissue. As can be seen in Figure 4.3 similar levels of BCRP protein were present in all 3 tissues.

Figure 4.1

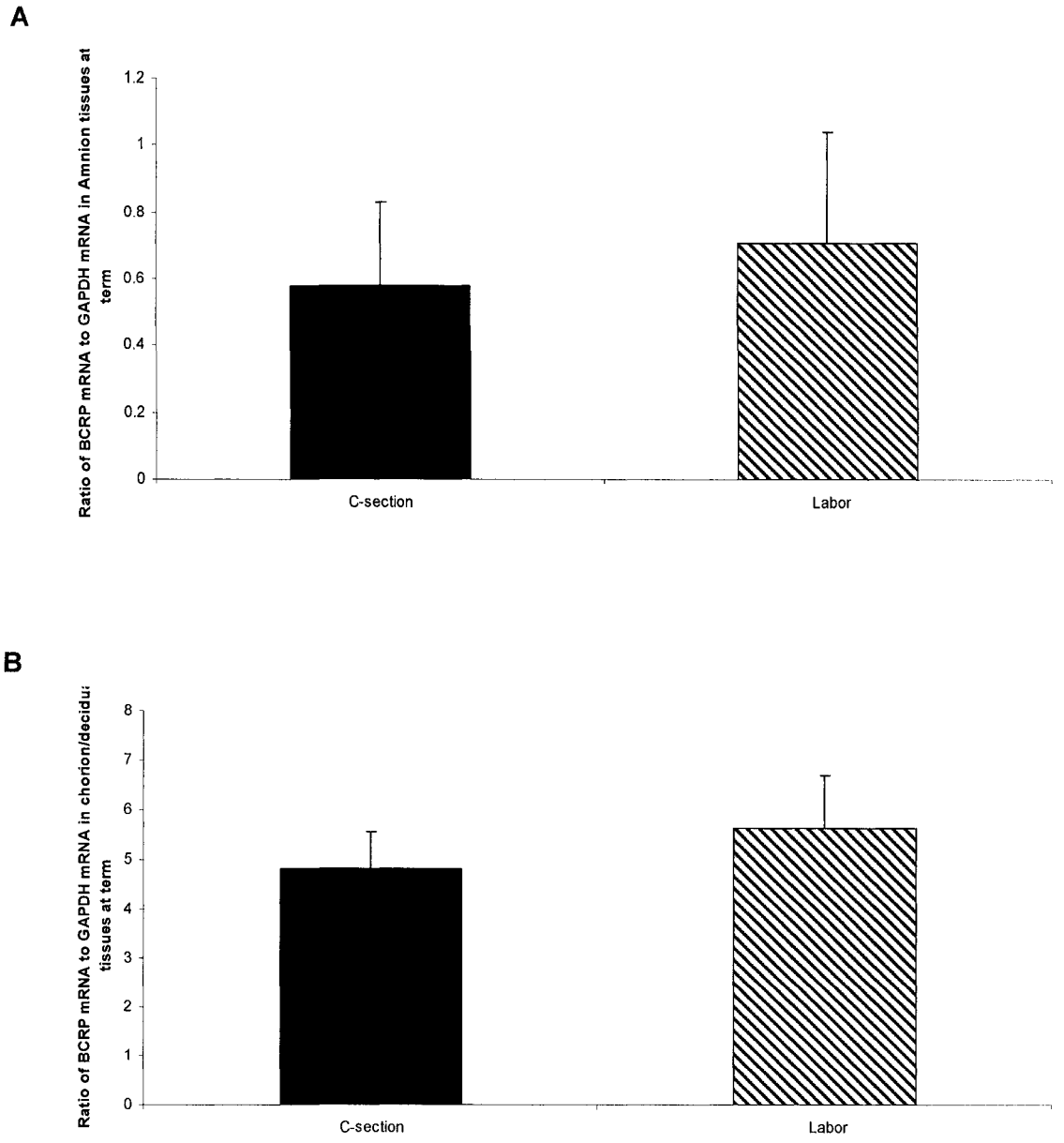


Figure 4.1: Quantitative real time RT-PCR of BCRP mRNA in human fetal membranes. Comparison of the expression of BCRP mRNA in amnion (**A**) and chorion/decidua (**B**) tissues before and after labor. Values shown represent mean ±SEM of the ratio of BCRP mRNA to GAPDH mRNA. Term (38-41wks) before (n = 12) and after labor (n = 12). P>0.05

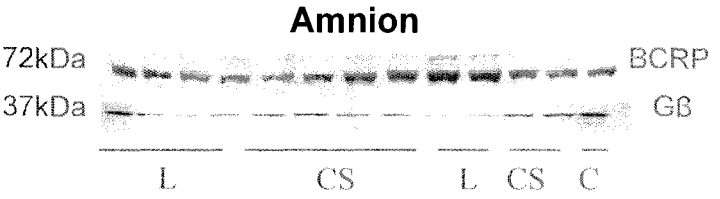
Figure 4.2: Western analyses of BCRP in term human fetal membranes tissues.

A and C: Typical Western blot analyses with BXP-21 antibody shows the presence of BCRP, 72kDa protein, in amnion and chorion/decidua tissues from C-section (CS) and labor (L). Gβ was used as loading control. Placenta was used as the tissue control C

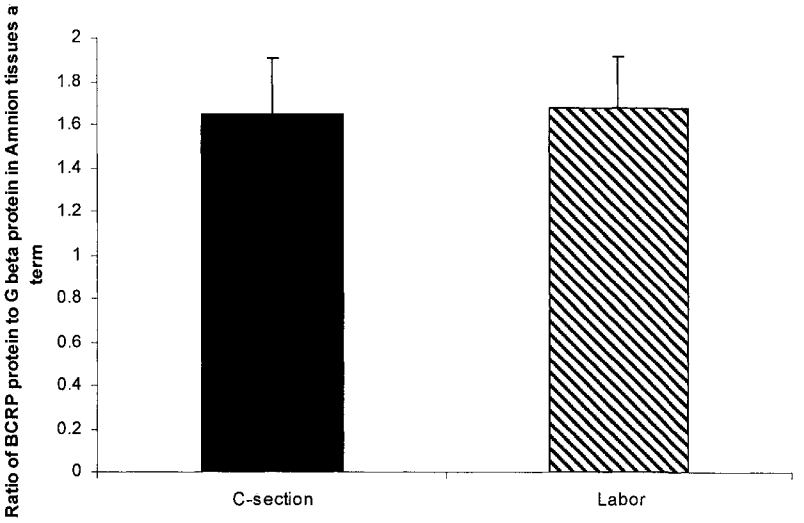
B and D: Quantification of BCRP protein in human fetal membranes by densitometry. BCRP protein levels did not change during labor in amnion (B) and chorion/decidua (D) tissues. In each group n = 12 tissues

Figure 4.2

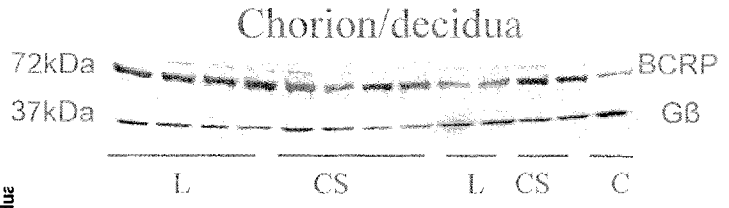
A



B



C



D

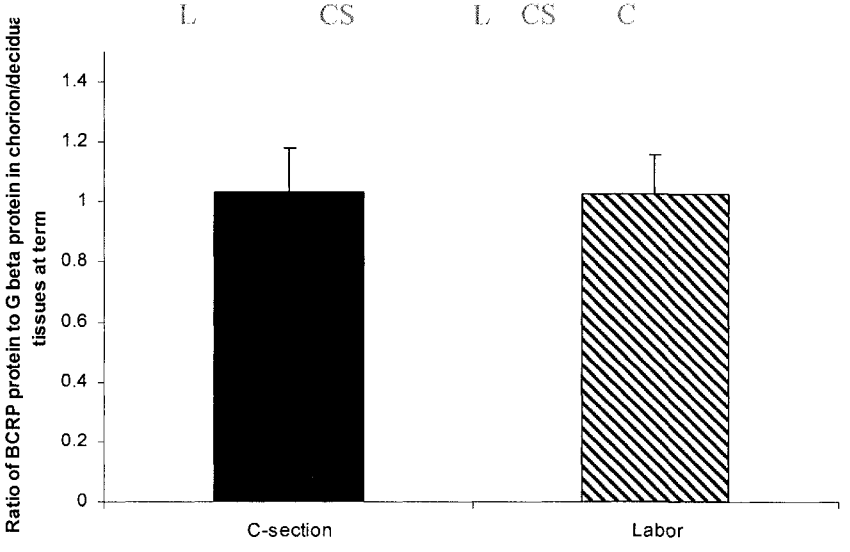


Figure 4.3

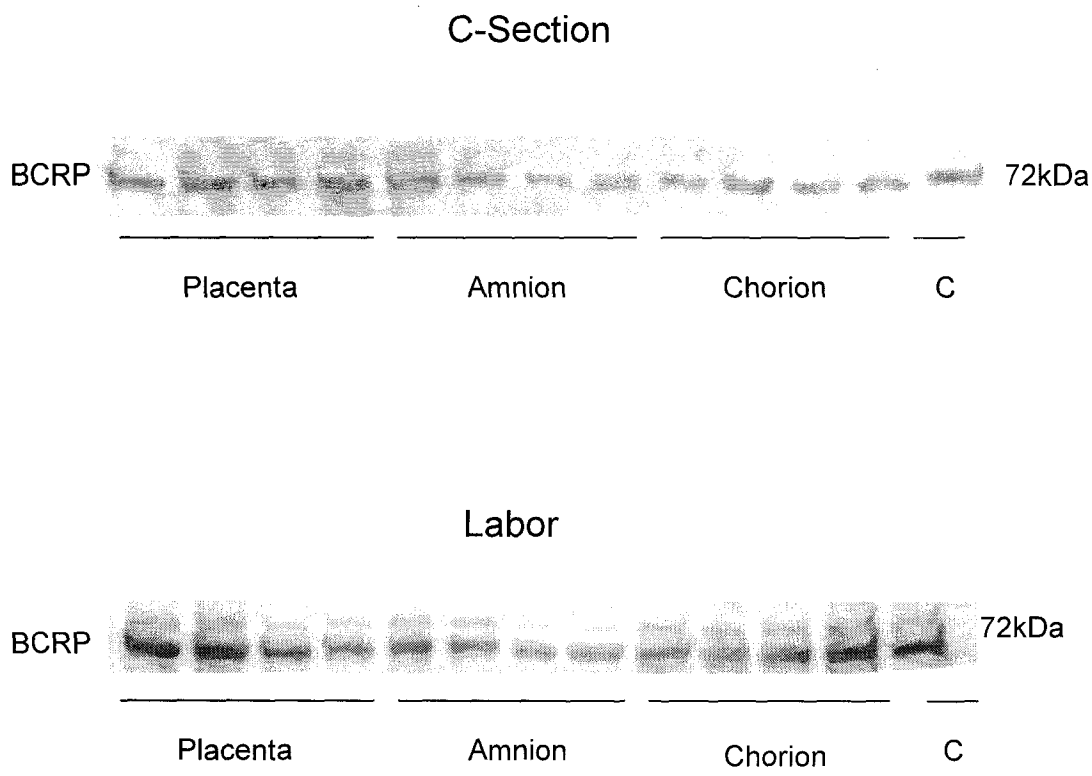


Figure 4.3: Western analyses of BCRP in term human placenta and fetal membrane tissues.

Western analysis carried out with the same amount of protein extracted from the amnion, chorion/decidua and placenta obtained by C-section prior to labor and following labor at term. Similar levels of BCRP protein were found in all 3 tissues with the same molecular weight. Placenta was used as the tissue control C.

BCRP protein was also localized in the fetal membranes (amnion and chorion leave) and decidua using immunohistochemistry. The protein was present in the amnion epithelial cells, in the chorion and also in the decidua (Figure 4.4*a*). However, it was absent from the mesenchymal cells situated between the amnion epithelium and chorion laeve trophoblast (Figure 4.4a and *b*). In the amnion epithelial cells BCRP was observed throughout the cytoplasm, and in some cells, it was highly expressed on the apical surface facing the amniotic fluid (Figure 4.4*b*), while in other cells it was highly expressed at the cell-cell junction between the epithelial cells (Figure 4.4*c*). In the chorion, BCRP was present on the plasma membrane and cytoplasm of the trophoblast cells (Figure 4.4*d*). In many of the trophoblast cells BCRP was primarily localized to the nucleus (Figure 4.4*e*). BCRP was also present in the decidua. Here the protein was located in the plasma membrane of the stromal cells (Figure 4.4*f* and *g*) and was also highly expressed on the endothelial cells of maternal blood vessels (Figure 4.4*h*). No staining was seen in control slides treated with pre-immune mouse IgG (Figure 4.4*i* and *j*). Figures 4.4g and j were not counterstained. There was no difference in the staining pattern of tissues obtained prior to or following labor.

Figure 4.4: Immunohistochemical localization of BCRP in term human fetal membranes.

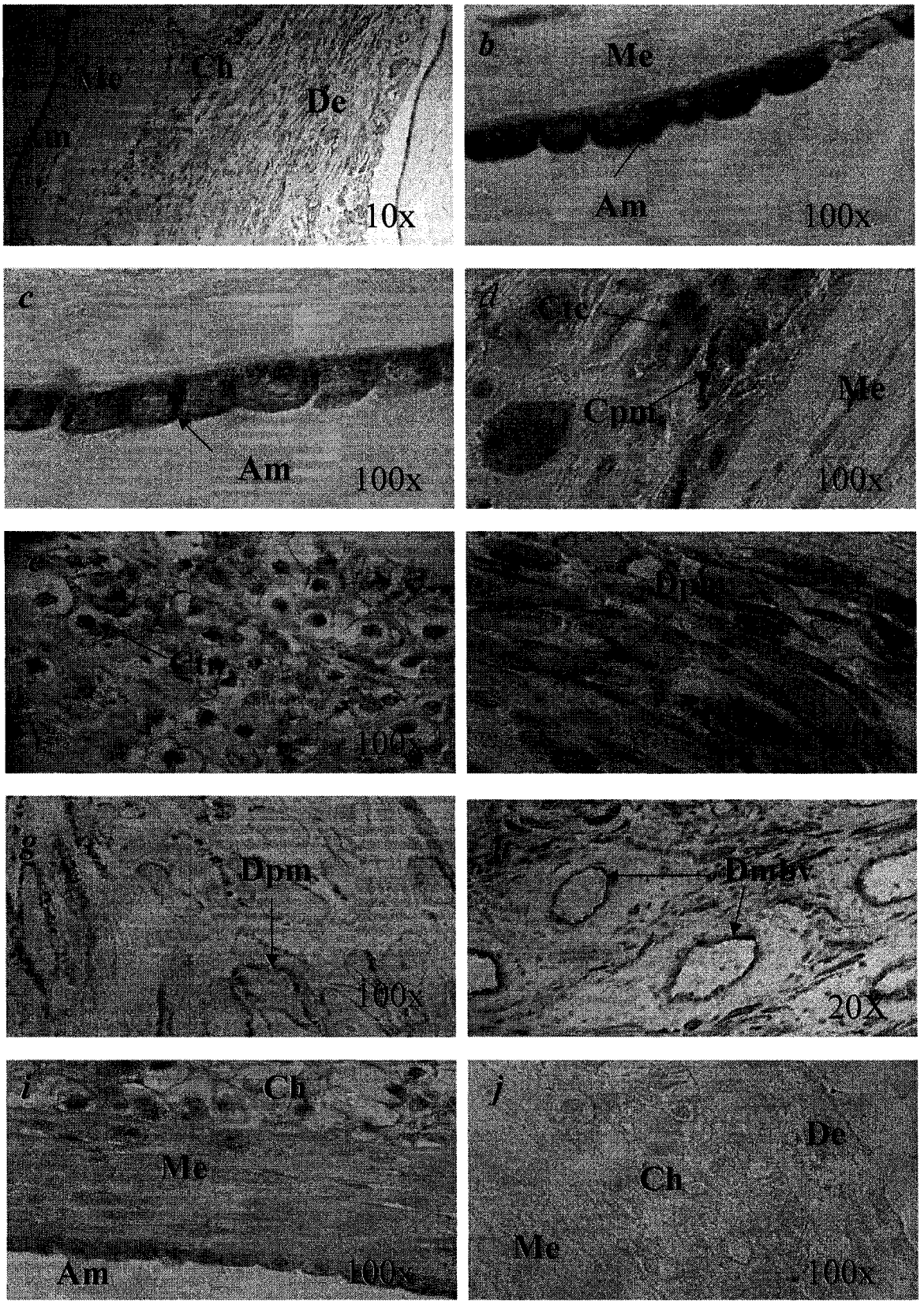
BXP-21, monoclonal antibody was used in dilution 1:100.

BCRP was present in the amnion (Am), chorion (Ch) and the decidua (De) but could not be detected in the mesenchymal layer (Me) between the amnion epithelium and the chorion leave trophoblast **(a and b)**. In the amnion epithelial cells BCRP was localized in the cytoplasm on the apical membrane (**b**) as well as between certain cells (**c**).

In the chorion BCRP was present in the chorion trophoblast cytoplasm (Ctc) and plasma membrane(Cpm) and was particularly evident in the nucleus (Ctn) of some cells (**d and e**). In the decidua BCRP was localized to the decidual plasma membranes (Dpm) and to the endothelial cells of some maternal blood vessels (Dmbv) **(f, g and h)**. No signal was obtained when tissues were incubated with non-immune mouse IgG **(i and j).**

a,b,c,d,e,f, h and i tissues were counterstained. Tissues shown in **g** and **j** were not counterstained

Figure 4.4


Ch
De
10x
b
Me
Am
100x
c
Am
100x
d
Cpm
Me
100x
100x
Dpm
100x
20X
i
Ch
Me
Am
100x
j
De
Ch
Me
100x

4.5 Discussion

This study demonstrates that BCRP is expressed in term human fetal membranes and the decidua in a cell and tissue specific fashion. In the amnion, it was localized specifically to the epithelial cells and was absent from cells in the mesenchymal area between the amnion and chorion trophoblast. In the chorion laeve cells BCRP was often localized to the cell nucleus and was also present in cytoplasm and plasma membrane. In the decidua BCRP was expressed on the plasma membrane of the stromal cells and was also particularly evident in the endothelial cells of maternal blood vessels. Protein levels in the amnion and chorion/decidua were similar to those found in the placenta. Comparison of both message and protein levels of BCRP in fetal membranes before and after labor and its cellular localization showed no significant difference.

The molecular weight of the BCRP protein in amnion and chorion/decidua of approximately 72kD is the same as that found in placenta. Also, a protein with a slightly higher molecular weight of approximately 75kDa was detected in the amnion and chorion/ decidua, which is similar to what we have found in the placenta[18]. It has been suggested by others that this might represent a glycosylated form of BCRP[32].

Both BCRP protein and message are found in the human placenta from very early stages of pregnancy to term[17,18] and the present report clearly demonstrates that BCRP is highly expressed throughout the fetal membranes (amnion and chorion laeve) and the maternal decidua at term. BCRP protein levels in the membranes were similar to that found in the placenta, which contains high levels of the protein. This would be consistent with the

transporter having an important role in these tissues. Previous studies in the placenta and other tissues have shown that BCRP is expressed in certain blood vessels[12, 13, 18]. The localization of BCRP to the plasma membrane in decidual stromal cells and the endothelial cell of maternal blood vessels underline the potential importance of BCRP in limiting the entry and exit of compounds from maternal blood into the pregnant uterus.

The cytoplasmic localization of BCRP in the amniotic epithelial cells as well as chorion trophoblast is consistent with previous studies indicating that the protein is shuttled to the cell membrane by cytoplasmic organelles and this may be important in the regulation of BCRP[33]. This localization of the protein has also been reported to occur in short-term folate deprivation of cells[34] and for mutant forms of the protein[35]. Aye et al also reported the cytoplasmic localization of BCRP in amnion epithelial cells. In primary cultures of amnion cells, these authors also demonstrated that inhibition of BCRP function by GF120918 led to an increase in cellular drug accumulation[25], showing that BCRP functions in the extrusions of drugs and toxins from the amnion cells. In our study we found that BCRP was also highly expressed at the junction of amnion epithelial cells indicating that it may be involved in intercellular communication, a function that will require further study.

In chorion laeve cells, BCRP was expressed in the plasma membrane and cytoplasm as expected, but was also localized to the nucleus. Nuclear expression of BCRP is intriguing and this localization has, to our knowledge, not been described previously. It is not clear

what function BCRP performs in the nucleus but the finding raises the possibility that it may modulate access of substances to the nuclear compartment.

Recent studies have shown that BCRP transports sulphated estrogens[36]. Under normal conditions sulfated estrogens are also found in amniotic fluid[37] and have been shown to act as precursors for estrogen within the membrane. Therefore the high expression of BCRP in the amnion epithelial cells, particularly on the apical membrane, may limit the entry of sulfated estrogens into the amnion epithelial cells and be involved in the regulation of estrogen formation in this tissue.

Cytokine production within the fetal membranes changes during labor[27,28, 38] and cytokines have been shown to alter BCRP expression in other tissues including placental trophoblast[23,29]. However, in the present study no difference in BCRP expression occurred following labor despite these reported changes in cytokine production. The present data however, cannot exclude the possibility that there were cell-specific changes within these tissues. Future studies using cells isolated with laser capture microdissection would be required to investigate this possibility.

Studies with human cancer cell lines suggest that BCRP expression may be regulated by estrogen[39-41] and progesterone[41]. Also, in placentally derived BeWo cells, estradiol down regulated BCRP expression while progesterone increased BCRP expression. Although when estrogen and progesterone were incubated together with these cells BCRP expression was increased[41]. Estrogen mediated down regulation of BCRP has also been reported in cytotrophoblast cultures[23]. From mid-gestation to term, the levels of

estrogens[42] and progesterone in maternal serum increase. Also, the fetal membranes have the ability to interconvert estrone and estradiol and this reaction is directed towards estrone production before labor and estradiol production after the onset of labor[42]. Our results indicate that any potential changes in estrogen levels in the tissues, which might occur during labor, did not alter BCRP expression during labor.

In conclusion, we have shown that BCRP is highly expressed in the human fetal membranes and decidua. We have described the cell specific localization of BCRP and shown that labor at term does not influence BCRP expression in these tissues. These findings raise the possibility that in addition to the placenta, BCRP in fetal membranes and the decidua may play an important role in the physiological function of these tissues.

Reference list for chapter 4 (page 128-131)

Chapter 5

Effect of cytokines and hypoxia on BCRP expression in human placenta

Authors: Yeboah D[1], Matthews SG[3], Gibb W[1,2]

[1]Department of Obstetrics and Gynecology and Cellular and Molecular Medicine, University of Ottawa, [2]Ottawa Health Research Institute, and [3]Departments of Physiology, Medicine and Obstetrics & Gynecology, University of Toronto

Reference: Manuscript not yet submitted.

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5.1 Abstract.

Multidrug resistance ABC proteins are highly expressed in barrier tissues. These include the intestines, blood brain barrier and the placenta. In these tissues these proteins are believed to limit the accumulation of drugs, xenobiotics and toxins. Breast cancer resistance protein (BCRP) and P-glycoproteins (P-gp) are abundantly expressed at the apical surface of the placental brush border membrane composed of syncytiotrophoblast cells. It has been reported that P-gp expression decreases towards term. BCRP expression however, increases towards term. It is not known what factors regulate BCRP expression in syncytiotrophoblast cells of the placenta. However, in other tissues and cell types it has been reported that BCRP expression and function may be regulated by cytokines and hypoxia. A number of pathological pregnancies result in placental hypoxia and/or increased production of cytokines by placental tissue. In this study we examined the effect of TNFα and IL-1β on BCRP expression in syncytialized trophoblast cells. We also studied the effect of hypoxia on BCRP expression by these cells. Our results determined that syncytial BCRP expression was not affected by increased levels of TNFα and IL-1β or by a reduced oxygen environment.

5.2 Introduction

ABC drug transporters are highly expressed in barrier tissues such as the intestines, blood brain barrier and the placenta (Peng *et al.*, 1999;Jonker *et al.*, 2000;Maliepaard *et al.*, 2001). Breast cancer resistance protein, BCRP, and P-glycoprotein (P-gp), are the two most abundantly expressed ABC drug transporters on the apical surface of placental syncytiotrophoblast (Evseenko *et al.*, 2006a;Aye *et al.*, 2007). BCRP is also present on endothelial cells of some fetal blood vessels (Yeboah *et al.*, 2006). These transporters extrude xenobiotics and some endogenous compounds such as steroids (Dean *et al.*, 2001;Janvilisri *et al.*, 2003;Schmitz & Kaminski, 2001) and by so doing; regulate the accessibility of such compounds to the placenta and fetus.

Over the past few years studies have shown that the level of BCRP in the placenta is either maintained throughout gestation or, increases at term (Mathias *et al.*, 2005;Yeboah *et al.*, 2006). Also, it has been shown that syncytiotrophoblast cells express higher levels of BCRP than cytotophoblast (Evseenko *et al.*, 2006b;Meyer zu Schwabedissen *et al.*, 2006). This suggests that factors associated with trophoblast differentiation may influence BCRP expression. However, information is limited on how BCRP expression in the placenta is regulated.

In other tissues, inflammation, hypoxia and steroids have been shown to alter BCRP expression. For instance, active inflammation of the gut mucosa of patients with ulcerative colitis leads to a significantly reduction of levels of BCRP and other multi-

drug resistance proteins (Englund *et al.*, 2006). Hypoxia has been shown to increase BCRP expression in the human placentally derived cell line, JAR cells, and also in bone marrow stem cells (Krishnamurthy *et al.*, 2004) and this could be the reason for high expression found in endothelial cells of veins, but not arteries, of the heart (Maliepaard *et al.*, 2001).

Inflammation and/or hypoxia are important in a number of pathological pregnancies. Under pathological conditions such as infection, preeclampsia and gestational diabetes, the placenta produces high levels of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interleukin 1 beta (IL-1β)(Roberts & Lain, 1998). Also, often under these pathological conditions, especially in preeclampsia, the amount of oxygen available to the placenta is lower than normal (Brosens *et al.*, 1972;Jauniaux *et al.*, 2006;Lunell *et al.*, 1984). These pathologies therefore have the potential to alter the expression and activity of drug transporters. Whether these conditions potentially make fetuses more prone to the accumulation of drugs and toxins has not yet been evaluated.

Studies on the regulation of BCRP expression in human placenta tissues or placentally derived cell lines are limited. Recent studies by Wang et al have shown that BCRP expression in BeWo cell lines is regulated by 17β estradiol and progesterone (Wang *et al.*, 2006) and a very recently published study examined the effect of steroids and cytokines in primary cultures of human trophoblast cells (Evseenko *et al.*, 2007b). In this latter study cytokines were found to decrease BCRP expression. The experimental protocol of the study was such that the effect of cytokines was examined during

syncytialization of the cytotrophoblast cells, a time when BCRP expression has been shown to increase (Meyer zu Schwabedissen *et al.*, 2006). In the present study we set out to examine the effect of cytokines and hypoxia on BCRP expression in placental trophoblast cells once they had syncytialized and were expressing high levels of the transporter.

5.3 Materials and methods

Tissue collection

Term (38–40 wk gestation) placenta tissues were collected immediately after elective C-section prior to labor from patients with no clinical evidence of infection at the Ottawa General Hospital with the approval of the ethics committee.

Tissue culture

After rinsing pieces of tissue in 0.9% cold saline solution to remove blood cells, trophoblast cells were isolated using Kliman's method (Kliman *et al.*, 1986) with modification (Premyslova *et al.*, 2003). Briefly, approximately 60g of tissue were coarsely minced and subjected to three 30min trypsin digestions at 37°C. The digest buffer, 0.125% trypsin and 0.02% DNase (Sigma-Aldrich MO, USA) in Dulbecco's modified eagle's medium, DMEM (Wisent Co. QC, Canada), was prepared immediately before each digest. Enzyme activity was halted with newborn calf serum (6.7% v/v) (Wisent) following digestion. Cells were pooled and loaded onto a 5 to 70% Percoll (Sigma) density gradient with a step increment of 5% Percoll. This was centrifuged for 20min at 2500xg. Trophoblast cells which sediment between 1.049 and 1.062g/ml were collected washed and cultured in 6-well plates at $6.5x10^5$ cells/cm^2 in DMEM supplemented with 10% Fetal Bovine Serum, FBS (Wisent Co.), 1% antibiotic-antimycotic (Sigma) solution and 1.5% Fungizone (GIBCO Auckland, NZ). Cells were incubated at 37°C with 5%CO_2 and 95% air for 72hrs. Media was replenished after every 48hrs. For cytokine studies 1ng/ml and 10ng/ml of TNFα and IL-β1 were introduced into culture medium after 72hrs of incubation. Proteins and mRNA were isolated after 24hrs

of treatment. For hypoxic studies cells were incubated at 37°C with 5%CO^2, 3%O_2 and 95%N_2 for 48hrs following the initial 72hr incubation at 37°C with 5%CO_2 and 95% air. Cells from individual tissues were cultured in triplicate for all treatments.

Western blotting

Cells were washed twice in 2.5mls of ice cold Phosphate Buffered Saline, PBS, containing 2.67mM KCl (Aldrich WS, USA), 1.48mM KH_2PO_4 (Aldrich WS, USA), 13.7mM NaCl and 8mM Na_2HPO_4 (BDH Canada). Cells were lyzed in the culture dish with 500µl of mammalian protein lysis buffer suplemented with Benzone and protease inhibitors (Qiagen ON, Canada). Cells were then scraped, collected into centrifuge tubes and set onto a shaker for 5min at 4°C. Following centrifugation for 20min the supernatant containing the protein extract was collected and protein concentration was determined with the BioRad protein assay reagent (BioRad, Richmond, CA, USA). Duplicates of samples containing 10µg of protein were diluted in an equal volume of Laemmli sample buffer (BioRad) and separated on 4-15% polyacrylamide gels (BioRad). Proteins were then transferred onto pure nitrocellulose blotting membrane (VWR international) by electrobloting at 100V for 1hr. Non-specific binding was blocked overnight with 5% non-fat dry milk (BioRad) in PBS containing 0.05% Tween (BioRad). Membrane was incubated overnight at 4°C with a mouse monoclonal anti human BCRP antibody, BXP-21, obtained from Calbiochem CA, USA (1:250) or an equal concentration of a non-immune mouse IgG for control. This was followed by incubation with HRP-conjugated sheep anti mouse IgG (1: 1000, 1hr; Amersham, UK). Proteins were visualized by chemiluminescence detection with the ECL system (Amersham) and Kodak BioMax

MR-1 film (Amersham,). Bands were quantified by densitometry with Gβ (T-20; Santa Cruz Biotechnology CA) as an internal loading control (1:5000) (Sun *et al.*, 2006;Yeboah *et al.*, 2006).

Immunohistochemistry

Slides were rinsed for 5min in PBS twice then fixed with 10% buffered formalin phosphate for 5min. This was followed by two 10min washes in PBS and dehydration in 75% ethanol for 5min. Slides were stored at 4°C in 95% ethanol until needed. Immunohistochemical studies were carried out as previously indicated (Sun *et al.*, 2006;Yeboah *et al.*, 2006). Breifly, cells were re-hydrated in 75% and 50% of ethanol for 5min each. Slides were then rinsed in H_2O then washed in PBS for 5min twice. Endogenous peroxidase activity was blocked with incubation in 0.3% (v/v) H_2O_2 (BDH England) in methanol (EMD NJ, USA) for 30min. Slides were washed for 10min in PBS (2X) and incubated in 1.5% of normal horse serum (Vector Laboratories, CA) in PBS for 20min to block non-specific binding sites. Cells were then incubated with 1:5000 v/v cytokeratin (Dako CA, USA) or 1:5000 v/v Vimentin (DakoCytomation, Denmark) or an equivalent amount of non-immune mouse IgG for 1h at room temperature. After washing in PBS for 5min (2X), slides were incubated with a rabbit anti mouse biotinylated antibody for 30min then washed and incubated with an avidin-biotin complex, all from the Vectastain Elite ABC kit (Vector Laboratories, CA) for 30min. Proteins were visualized using DAB peroxidase substrate (Sigma). Slides were counterstained with haemotoxylin (EMD), dehydrated in ethanol (EM Science), cleared in xylene compound (EM Science) and mounted with permount (Fisher Scientific NJ, USA).

Real time RT-PCR

Total RNA was extracted from cells using the Qiagen RNeasy Mini kit and DNase treated using the RNase-free DNase set (Qiagen, ON Canada). Real time RT-PCR was done in one step using the TaqMan One-Step RT-PCR Master Mix Reagents Kit and the ABI PRISM 7000 apparatus (Applied Biosystems, CA USA) according to manufacturer's instruction. As in previous studies (Yeboah *et al.*, 2006) primers and probe sequences for BCRP were designed with the Primer Express 2.0 software (Applied Biosystems) and checked with BLAST to minimize cross reactivity with other sequences. The following sequences were used: BCRP forward 5'-GGCTTCAGTACTTCAGCATTCCA-3', BCRP reverse 5'-AGTCCTGGGCAGAAGTTTTGTC-3' and hybridization probe for BCRP 5'-FAM-ATGGATTTACGGCTTTGCAGCATAATGAATTTTT-TAMRA-3'supplied by Applied Biosystems. The PCR product was sequenced to confirm that the expected sequence was amplified. Glyceraldehyde-3-phosphate dehydrogenase, GAPDH was used as the internal control. GAPDH control reagents including primers and probe were designed and supplied by Applied Biosystems (product number 402869). The one-step RT-PCR was done in a 20μl reaction containing 1x TaqMan Master Mix, 1x MultiScribe and RNase inhibitor Mix, 100ng of RNA, 0.125μM of each primer and 0.2μM of probe for BCRP. For GAPDH 0.25μM of each primer and 0.125μM of probe were used. Reverse transcription was performed at 48°C for 30min and stopped at 95°C for 10min followed by 40 cycles of amplification at 95°C for 15 sec and 60°C for 1 min. A standard curve was constructed with serial dilutions of RNA for analysis of the copy number of

the target and the control genes in each sample. The results were normalized using the ratio of the target DNA concentration to that of the control DNA (GAPDH).

5.4 Results

Immunohistochemistry of the trophoblast cell cultures following 72hr of culture demonstated that the cells had syncytialized during culture as shown by cytokeratin staining of multinucleated cells (Figure 5.1). Using trypan blue exclusion the cells were consistently found to be > 90% viable. Cells were originally cultured for 3 days to allow syncytialization and then exposed to cytokines or hypoxia.

Real-time PCR was used to quantify BCRP mRNA in cultures treated with TNFα or IL1β for 24h. Neither TNFα nor IL1β, at concentrations of 1ng/ml and 10ng/ml had any significant effect on BCRP mRNA expression (Figure 5.2A and B). Similarly, Western analysis revealed no difference in BCRP protein levels in response to treatment with these cytokines (Figures 5.3 and 5.4).

Following exposure of cells to 3% oxygen for 48 hours BCRP mRNA levels dropped to about 50% of control cultures maintained in 20% oxygen (Figure 5.5). However, Western analysis demonstrated that the decrease in mRNA observed under hypoxic conditions did not affect BCRP protein levels (Figure 5.6A and B).

Figure 5.1

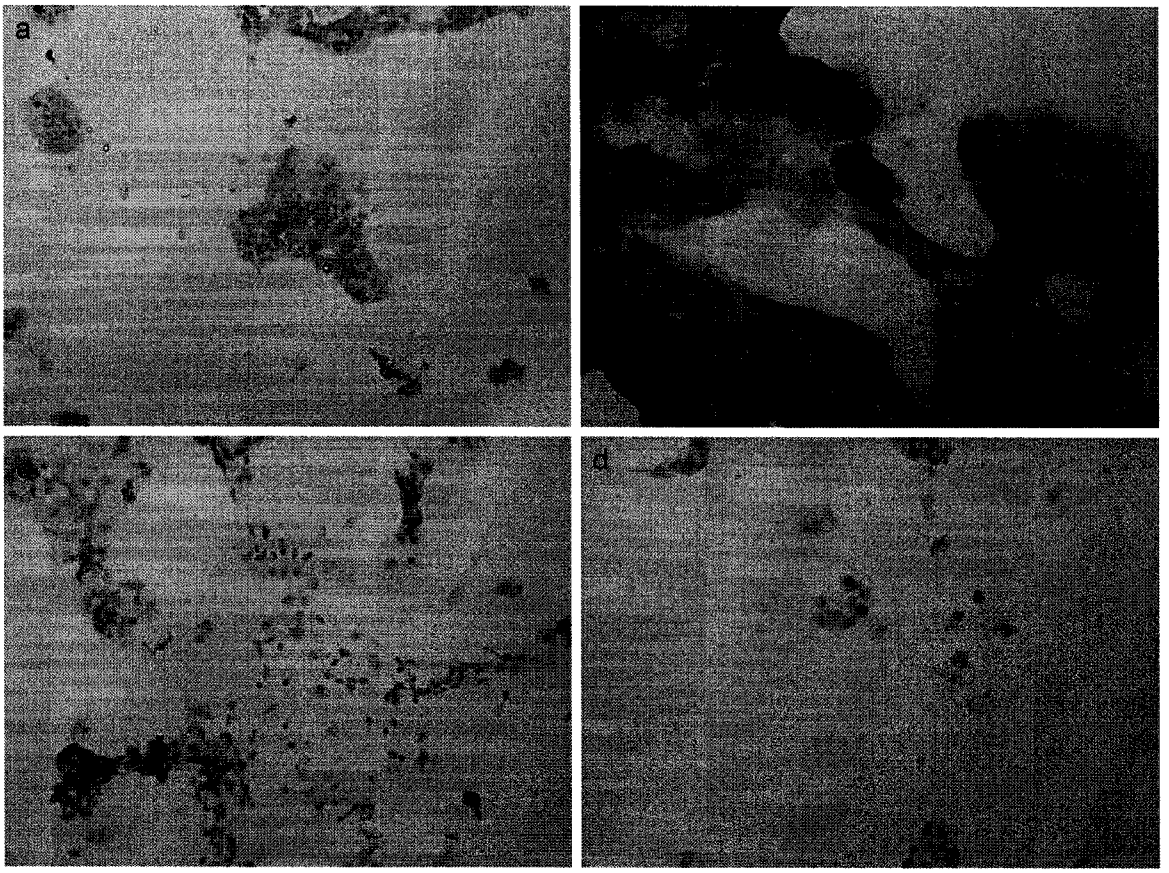

Figure 5.1: Immunohistochemical staining of differentiated trophoblast cells 48-72hrs in culture.

Cytotrophoblast cells (unicellular) differentiate to form multinucleated syncytiotrophoblast cells after 2 to 3 days in culture. Figures 1a and b show multinucleated cells stained positive for cytokeratin which is expressed by all epithelial cells including trophoblast cells. The negative control slides 4c and d show staining with non-immune mouse IgG and vimentin respectively. Trophoblast cells do not express vimentin.

Figure 5.2

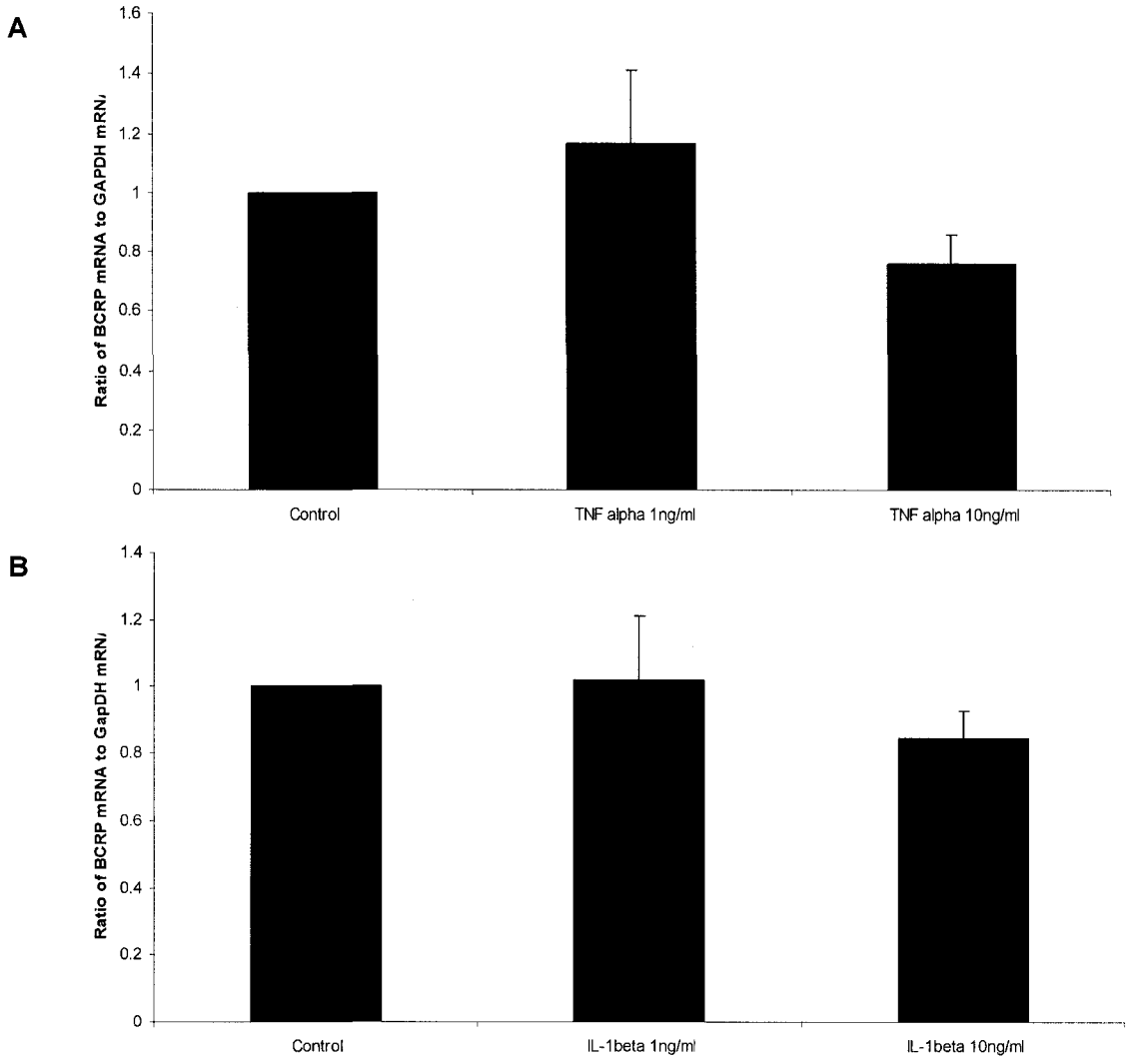

Figure 5.2: Expression of BCRP mRNA in syncytiotrophoblast cells following treatment with TNFα and IL-1β.

Comparison of the expression of BCRP mRNA in syncycialized cells following treatment with TNFα (A) and IL-1β (B) (1ng/ml and 10ng/ml). Control cultures were not treated. Values shown represent mean ±SEM of the ratio of BCRP mRNA to GAPDH mRNA. Term (38-41wks) C-section tissue was used for culture. Results represent five different tissues with each treatment analyzed in triplicate. $P>0.05$

Figure 5.3

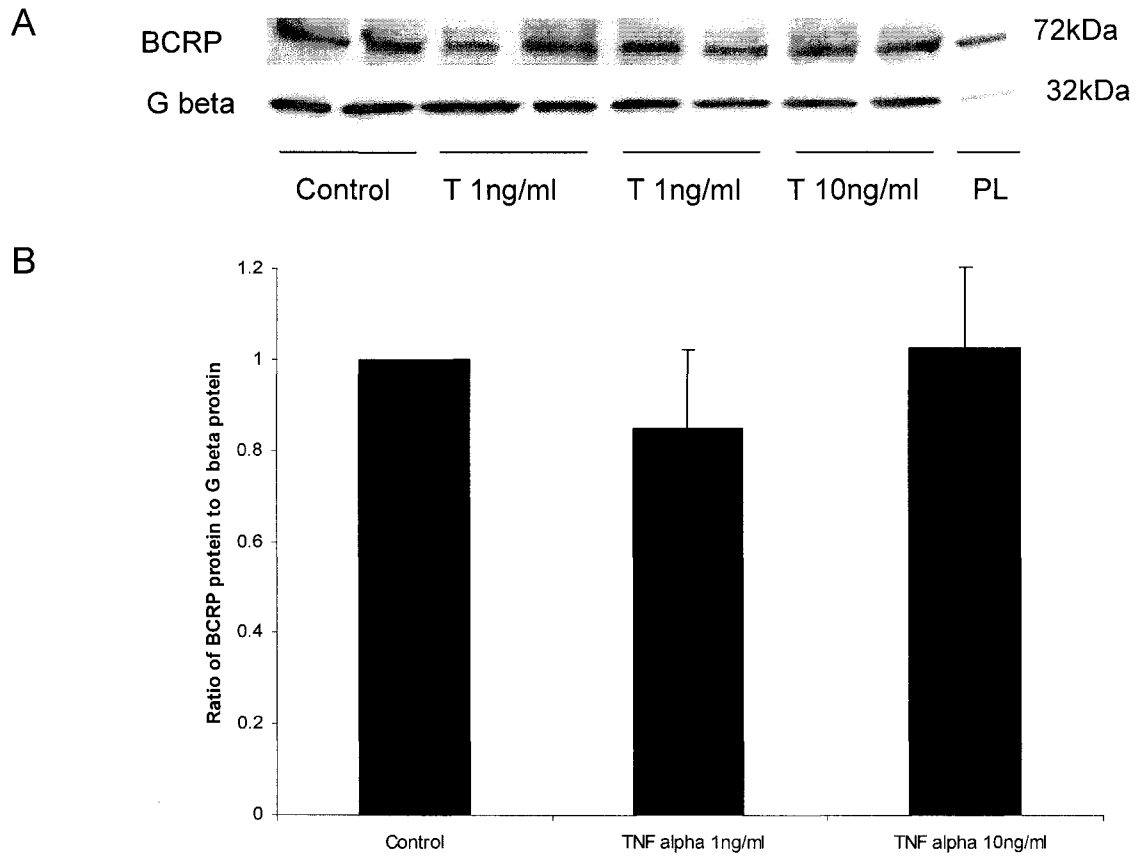

Figure 5.3: Western analysis of BCRP in syncytiotrophoblast cells in the presence of TNFα.

A: A typical Western blot of BCRP protein in differentiated trophoblast cells in culture treated with TNFα for 48hrs (T). The 72kDa protein was detected in all samples. Gβ was used as the internal or loading control. Also, proteins extracted from placenta tissue were run in each experiment as control (PL).

B: Quantification of protein levels by densitometry. BCRP protein levels in the syncytialized cells were quantified against Gβ protein levels. BCRP protein levels were not affected by cytokine concentration. Results represent five different tissues with each treatment analyzed in triplicate. $P>0.05$

Figure 5.4

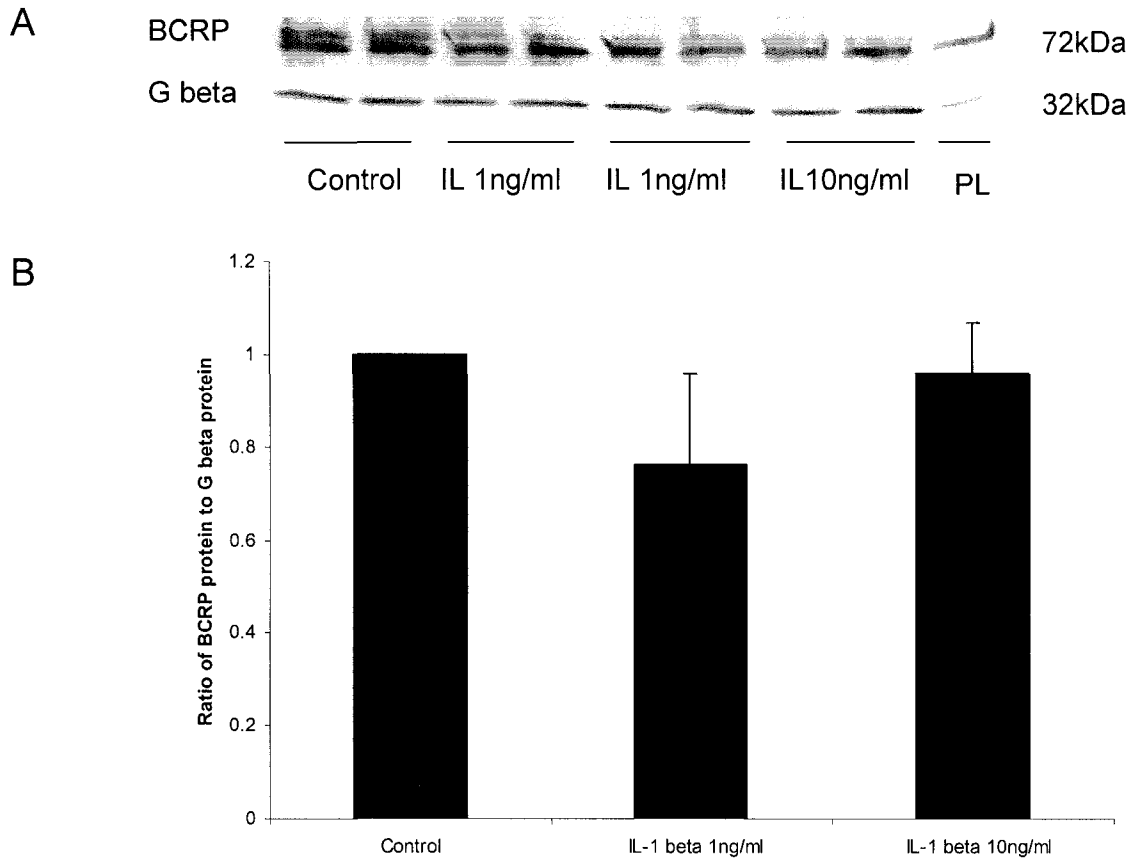

Figure 5.4: Western analysis of BCRP in syncytiotrophoblast cells in the presence of IL-1β

A: A typical Western blot of BCRP protein in differentiated trophoblast cells in culture treated with IL-1β for 48hrs (IL). BCRP protein was present in all samples. Gβ was used as the internal or loading control. Proteins extracted from placenta tissue were run in each experiment as control (PL)

B: Quantification of protein levels by densitometry. BCRP protein levels in the syncytialized cells were quantified against Gβ protein levels. BCRP protein levels were not affected by concentration of IL-1β. Results represent five different tissues with each treatment analyzed in triplicate. $P>0.05$

Figure 5.5

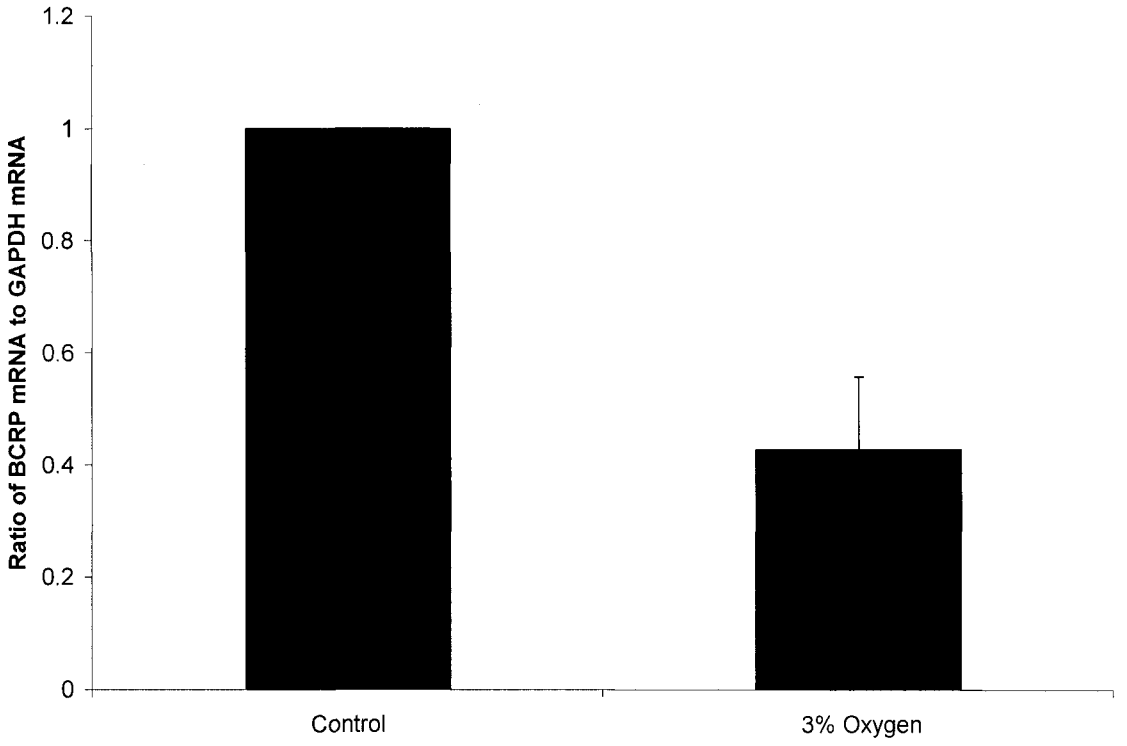

Figure 5.5: Expression of BCRP mRNA in syncytiotrophoblast cells following exposure to 3% oxygen for 48h.

Trophoblast cells cultured under normaxia (5% CO_2 and 95% air) for 72hrs to allow differentiation were either maintained in normaxia or hypoxia (3%O_2, 5% CO_2 and 92% N_2) for another 48h. Total RNA was extracted and BCRP levels in samples quantified by real-time RT-PCR. Following 48h of hypoxia BCRP mRNA levels in culture dropped to about 50% of that found in control cultures. Results represent six different tissues with each condition analyzed in triplicate. P=0.002

Figure 5.6

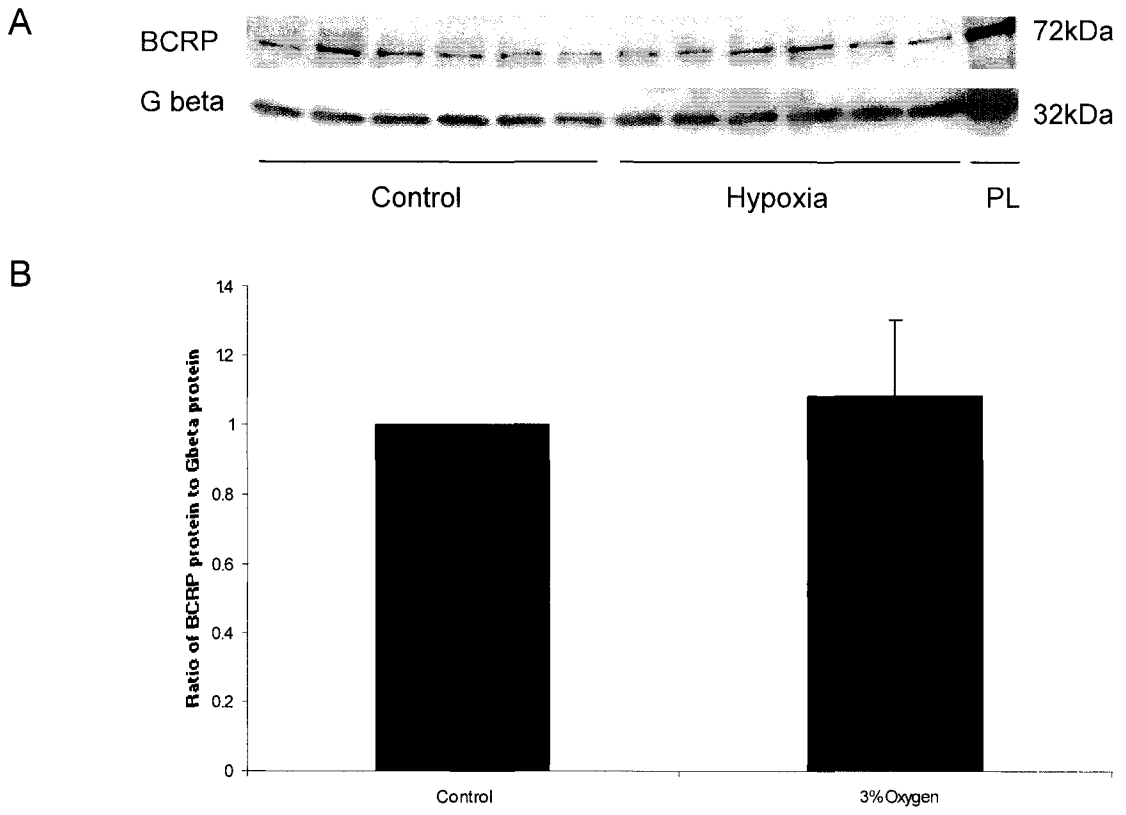

Figure 5.6: Western analysis of BCRP in syncytiotrophoblast cells following exposure to hypoxic conditions.

A: A typical Western blot of BCRP protein in differentiated trophoblast cells in culture exposed to 3% O_2 for 48h. Gβ was used as the internal or loading control. Proteins extracted from placenta tissue were run in each experiment as control (PL).

B: Quantification of protein levels by densitometry, (n = 6). BCRP protein levels in the syncytialized cells were quantified against Gβ protein levels. BCRP protein levels were not affected by the 48h exposure to hypoxia.

5.5 Discussion

We have demonstrated that TNFα and IL-1β did not alter BCRP mRNA expression or protein levels in human placental syncytiotrophoblast cultures. However, hypoxia decreased BCRP mRNA levels but did not alter BCRP protein levels.

Under normal conditions the placenta secretes basal levels of cytokines (Turner *et al.*, 2002). It has been reported that IL-6 knockout mice have abnormal placentation and parturition. IL-1β has also been implicated in implantation and normal labor (Bowen *et al.*, 2002;Turner *et al.*, 2002). Cytokine production by gestational tissues increases at labor whether at term or preterm (Saito *et al.*, 1993;Keelan *et al.*, 2003;Bowen *et al.*, 2002) and at preterm labor with infection, the cytokine levels are highly elevated (Tanaka *et al.*, 1998;Keelan *et al.*, 1999;Keelan *et al.*, 2003). Increased concentrations of pro-inflammatory cytokines may play also play a key role in cervical ripening (Winkler, 2003). Indeed cytokines including IL-1β and TNFα have been reported to increase in cervical tissues during labor (Winkler, 2003;Sennström *et al.*, 2000).

It has been demonstrated in numerous studies that isolated cytotrophoblast cells syncytialize following 48-72 hours of culture (Evseenko *et al.*, 2006b;Kliman *et al.*, 1986). Recent studies by Evseenko and colleges (Evseenko *et al.*, 2007b) on the effect of TNFα and IL-1β on cytotrophoblast cells during differentiation into syncytiotrophoblast showed a significant reduction in the levels of both BCRP mRNA and protein. This contrasts with the present study, which found no effects on BCRP levels in syncytiotrophoblast. During differentiation the expression of BCRP is increasing

markedly (Evseenko *et al.*, 2006b;Meyer zu Schwabedissen *et al.*, 2006) and in our system the BCRP is already highly expressed. The findings from both studies may therefore have important physiological implications. For example, in early pregnancy when the placenta is developing and there are higher concentrations of cytotrophoblast cells in the placenta, TNFα and IL-1β may have a marked impact on BCRP expression by the differentiating cytotrophoblast. Immunohistochemical studies of tissues from early gestation (6-13 wks) have shown that BCRP expression is localized to the syncytiotrophoblast (Yeboah *et al.*, 2006). Since these cells form the outer layer of placental tissue in contact with maternal blood, it may well be that cytokines in the maternal circulation in early pregnancy, do not have much of an effect on BCRP expression by the placenta as a whole. However, cytokine production within the placenta which may allow easy access to the cytotrophoblast cells undergoing differentiation could have a profound impact on BCRP expression. Later in pregnancy when there are fewer cytotrophoblast cells in the placenta (Hemmings *et al.*, 2001;Aplin, 1991) the impact of cytokines might be minimal therefore having little direct impact on the transport ability of the placenta via BCRP. The present findings are also consistent with our previous studies showing no change in BCRP levels in the placenta or fetal membranes during labor, a time when there is increased cytokine production within the intrauterine tissues (Saito *et al.*, 1993;Keelan *et al.*, 2003;Bowen *et al.*, 2002).

BCRP has been suggested to have a role in protecting stem cells from hypoxia. A side population (SP) cells of bone marrow hematopoietic stem cells express high levels of BCRP (Krishnamurthy *et al.*, 2004). It has been reported that BCRP transports

pheophorbide *a,* which is derived from dietary chlorophyll and is structurally similar to protoporphyrin IX, a heme precursor (Krishnamurthy *et al.*, 2004;Krishnamurthy & Schuetz, 2005). Heme accumulates in cells when oxygen levels are low. This heme build up is toxic to cells and can inhibit mitochondrial function which can lead to cell death (Böhm *et al.*, 2001;Sandberg & Romslo, 1980). It is believed that BCRP transports the heme precursors and thus prevents cell death. This gives stem cells the ability to survive in a low oxygen environment (Krishnamurthy *et al.*, 2004). Hypoxia has also been shown to increase BCRP expression in a number of other cells and tissues. For example, studies with JAR cell lines (placental choriocarcinoma) have shown an increase in BCRP expression under hypoxic conditions and an up-regulation of BCRP expression has also been reported in other cell lines of non-placental origin via the HIF1 pathway (Krishnamurthy *et al.*, 2004).

In the present study trophoblast cells were cultured under normoxic conditions for 72 hrs prior to hypoxia as it has been shown previously that hypoxia inhibits syncytialization of the cytotrophoblast cells (Kudo *et al.*, 2003). Cells were then maintained under hypoxic conditions (3%O_2) for 48hr. A significant reduction in BCRP message levels was observed, however, protein levels were unaffected by the reduced oxygen environment. These results were surprising, as an upregulation of BCRP expression has been reported in other systems as discussed above. The decrease in BCRP mRNA levels we observed in our study may suggest that BCRP mRNA in syncytialized trophoblast cells is unstable in a low oxygen environment or, that BCRP mRNA synthesis was affected by the reduction in oxygen levels. The absence of a significant effect on protein levels would suggest that

BCRP protein is very stable. Also, since the amount of BCRP mRNA and protein present in the syncytialized cells is high to begin with the effect of hypoxia on mRNA levels may require a longer time to affect the overall content of BCRP protein.

In conclusion, syncytotrophoblast cells in culture show high levels of BCRP mRNA and protein and it appears that the levels of BCRP protein are not affected by changes in the culture environment including increased levels of cytokines and reduced oxygen levels. If these studies were to be extended to the in vivo situation they would indicate that BCRP expression in the placenta is very robust and would be maintained even under adverse conditions found in a number of pathological pregnancies. This study indicates that the expression of the protein is very stable and may imply that BCRP is essential for normal placental function as well as being involved in transport functions. This has indeed been proposed as recent studies have indicated that BCRP is important for cell survival during trophoblast differentiation. BCRP may prevent the progression of apoptosis during the transient phase of plasma membrane lipid and protein displacement associated with cell fusion (Evseenko *et al.*, 2007a).

Reference list for chapter 5 (page 131-135)

Final discussion

The goal of this thesis was to study the expression pattern of BCRP throughout pregnancy to determine if at certain periods during pregnancy the expression levels of BCRP mRNA and protein were altered. This might indicate that the fetus was more susceptible to the accumulation of toxins, xenobiotics and drugs during these periods. Studies with cell lines had suggested a regulatory role for both estrogen and progesterone in BCRP expression. Since these hormones are produced by the placenta and their levels increase with advancing gestation we proposed that BCRP expression pattern would change in response to these hormones.

When we embarked on the project there was very limited published data on the expression of BCRP in the human placenta. For the most part these studies had demonstrated the presence of BCRP in the human placenta with immunohistochemistry and western analysis (Jonker *et al.*, 2000;Maliepaard *et al.*, 2001a). During our study, Mathias et al published data on the expression of BCRP at various gestational ages (Mathias *et al.*, 2005). Their study concluded that BCRP expression in the placenta did not change during pregnancy. However, they focused on syncytial microvillous membrane levels of BCRP and only looked at a limited number of tissues. We examined BCRP expression in whole placental tissue and studied a large number of tissues from very early stages in gestation. By using whole placental tissue we were able to show that BCRP was expressed in fetal blood vessels as well as the syncytiotrophoblast.

Our data suggest that a post transcriptional up-regulation of BCRP expression may occur at term as protein levels at this stage in pregnancy were higher than those found in tissues from earlier stages of pregnancy whereas mRNA levels were unaltered. Immunohistochemical studies localized the protein to the placental barrier cells, the syncytiotrophoblast cells, and some fetal blood vessels. We reported for the first time that BCRP is expressed in the endothelial cells of some fetal blood vessels and the proportion of fetal blood vessels expressing BCRP appears to increase at term.

In recent studies trophoblast differentiation has been shown to correlate with an increase in BCRP expression (Meyer zu Schwabedissen *et al.*, 2006). A study of BCRP expression with primary cultures of cytotrophoblast cells showed an increase in BCRP levels (protein and mRNA) with differentiation of the mononuclear cytotrophoblast into the multinuclear syncytiotrophoblast (Evseenko *et al.*, 2006). Since there is an increase in syncytialization with advancing gestation, our data showing an increase in BCRP levels in late pregnancy would be consistent with these findings. Also, the increase in fetal capillaries expressing BCRP at term may contribute to the increase in BCRP levels in late pregnancy.

In future studies focusing on specific cells using the laser capture micro-dissection technique would be of considerable interest. By isolating the syncytiotrophoblast cells and the endothelial cells of fetal capillaries and studying BCRP protein and mRNA levels at early gestation and term we will be able to confirm that the

increase in BCRP levels we observed was due to an increase in BCRP expression in these cells specifically.

It has been reported in the cardiovascular system that BCRP is expressed in endothelial cells of veins but not arteries (Maliepaard *et al.*, 2001b). Also in other systems such as the submucosa of the stomach, the intestines and colon, BCRP has been located in venous capillaries (Maliepaard *et al.*, 2001b). In the placenta, fetal veins carry the oxygen rich blood from the placenta while arteries carry deoxygenated blood to the placenta and it is possible that the BCRP expression we observed was in the arteries. If indeed oxygen levels regulate BCRP expression in the endothelial cells of blood vessels then placental hypoxia would affect BCRP expression in fetal blood vessels. It would be of interest to examine this possibility by studying BCRP expression in fetal vessels of placentas from compromised pregnancies resulting in fetal hypoxia.

Once we had established that BCRP is expressed by the placenta throughout pregnancy our next aim was to examine whether BCRP was present in the attached fetal membrane tissues. No published data had reported the presence of BCRP in fetal membranes until as recently as May of this year (Aye *et al.*, 2007). These studies however focused on BCRP and other multi-drug resistant proteins expression in the amnion membrane. We studied both the mRNA levels and protein levels of BCRP in the amnion and chorion/decidua as the protein was localized to both membranes and we also studied the effect of labor on BCRP levels in the membranes. Our data showed that BCRP levels in the membranes did not change following labor. Even though the fetal

membranes produce increasing levels of cytokines during labor and cytokines have been reported to affect BCRP expression levels in other tissues it appears that placental membrane expression of BCRP is not affected by increased cytokine levels.

Functional studies of BCRP protein in the amnion epithelial cells demonstrated that BCRP is active in these cells (Aye *et al.*, 2007). These findings open the door for further studies on the function of BCRP in fetal membranes and how the expression of the protein is regulated in these tissues.

In future studies the investigation of the nuclear function of BCRP in chorion trophoblast cells would be of interest. We would confirm the presence of the protein with Western blot analysis of chorion trophoblast nuclear proteins. Once the presence of the protein had been substantiated we would then proceed to investigate the function of BCRP at this location. This localization of BCRP has not been previously reported therefore it is of great interest. Also, in future studies we would investigate the regulation of BCRP expression in the fetal membrane tissues. Since the protein was found to be differentially expressed in a tissue specific manner, it may be differentially regulated in the different tissues. The effects of cytokines, estrogen and hypoxia would be studied in primary amnion epithelial cell and chorion trophoblast cell cultures.

Currently we are the first group to have examined the effect of cytokines and hypoxia on BCRP expression in syncytialized trophoblast cells in primary culture. Until recently any in vitro work regarding placental BCRP studies have been carried out in

mice or cell lines of both human and mouse placental origin (Imai *et al.*, 2003;Krishnamurthy *et al.*, 2004;Wang *et al.*, 2006). Even though these have been valuable tools and have provided insightful information regarding this protein, there are some limitations on the interpretation and the implications of the data obtained to the human placenta. Although primary cell culture has its own limitations, which will be discussed later, primary culture of placental cells and explant cultures provide the closest tools for studying protein expression and function in the human placenta under controlled conditions.

Our study on the effect of hypoxia on BCRP expression is the first such study in primary trophoblast culture. Other studies have been done using other cell types and cell lines. Data gathered from our studies suggest a minimal effect, if any, on the protein levels of BCRP under hypoxic conditions. Since the results obtained from our studies are different from those reported using cell lines and stem cells, it may be worthwhile to examine the effect of hypoxia on undifferentiated unicellular trophoblast cells. This would provide information on the effect of placental hypoxia that occurs in early gestation on BCRP expression. Placental hypoxia can lead to many significant consequences on fetal growth and development and may lead to increased infant mortality and morbidity (Ferrario *et al.*, 2007;Perrone *et al.*, 2007).

Evidence suggests that cytokines may play an important role in the onset of labor (Romero *et al.*, 1989;Saito *et al.*, 1993) and the rupture of membranes (Menon & Fortunato, 2007;Moore *et al.*, 2006;Romero *et al.*, 1989). The levels of TNFα and IL-1β

in term placenta and fetal membrane tissues (Hanna *et al.*, 2000) increase at labor (Winkler, 2003;Sennström *et al.*, 2000;Sadowsky *et al.*, 2006). Englund G et al had reported that expression of BCRP in gut mucosa cells was affected by the presence of cytokines (Englund *et al.*, 2006). There have been no published reports on the effect of cytokines on the expression of BCRP and other drug transporters on syncytiotrophoblast cells of the placenta, epithelial cells of the amnion or the trophoblast cells of the chorion laeve.

Earlier this year, Evseenko et al published data on the effect of TNFα and IL-1β on BCRP and P-gp expression in trophoblast cells (Evseenko *et al.*, 2007). However their studies were carried out on undifferentiated unicellular trophoblast cells. Since the syncytialized trophoblast and endothelial cells of fetal blood vessels are the cells that express BCRP in the placenta and also since the syncytiotrophoblast are the blood-placental barrier cells we focused our studies on these cells. The absence of an effect of cytokines on BCRP expression in the syncytiotrophoblast, as our data shows, is noteworthy.

As we examined human tissues there were some limitations with tissue acquisition. We did not have access to tissues at certain periods of gestation. Also, although as mentioned earlier, primary cultures provide an excellent tool for studying normal protein expression and function in the body, the information these studies provide can be limited. First of all, the culture environment may not accurately mimic the

physiological environment in vivo, which may mean that certain substances required for the normal biological activities of the cells are absent (Milkiewicz & Haas, 2005).

We used an established cytotrophoblast culture procedure developed by Kliman (Kliman *et al.*, 1986), with some modification, for our in vitro studies. Kliman's method is the most commonly used procedure for primary trophoblast cultures. By this method, we isolated cytotrophoblast cells using a Percoll density gradient and tested the purity of our cultures with cytokeratin staining. However, we could not completely eliminate contamination by other cells. The placenta contains many different cell types and as such purifying cultures could be a challenge. To overcome the potential problem of contamination we extended our protocol to include an immunopurification step using mouse monoclonal anti human HLA class1 antibody. Villous trophoblast cells do not express this protein but all other cells of the placenta do (Douglas & King, 1989). However this further extended the already long protocol and led to a decrease in cell viability. The procedure was therefore discontinued. Most current studies continue to use the Percoll gradient as the only purification step and have demonstrated cell viability and cytotrophoblast differentiation.

The next step for our in vitro analysis would be functional studies. BCRP mediated transport of estrogenic compounds and BCRP substrates such as daidzein and genistein which are found in the amniotic fluid would be studied in primary amnion epithelial and trophoblast cultures. These studies would demonstrate the activities of BCRP in the placenta and amnion membrane with respect to these substrates and whether

BCRP might regulate the access of these compounds to and from the fetus via the amniotic fluid.

Also, BCRP mediated transport of DHEAS and estradiol would be studied. The placenta produces estrogens in large quantities during pregnancy (Byers *et al.*, 2007). DHEAS is a precursor of estrogen and the synthesis of estrogen by the placenta requires precursors from both the fetus and the mother (Pentti, 2005;Pentti, 1978). It has been reported that BCRP can transport sulphated estrogens in cell lines (Imai *et al.*, 2003;Suzuki *et al.*, 2003). However, no such studies have been reported in primary trophoblast cultures. The transport of DHEAS and estradiol by BCRP might suggest a regulatory role for BCRP in estrogen synthesis during pregnancy.

Finally BCRP mediated transport of folate would be examined. Folate is essential for methylation and gene silencing and is therefore very important in embryogenesis and fetal programming (Tamura & Picciano, 2006;Antony, 2007). Thus, it is crucial that the appropriate intracellular concentration of folate be maintained in the developing embryo/fetus for normal development. Folate deficiency in pregnancy leads to the development of spina bifida and other neural tube defects in fetuses due to the rapid rate of proliferation of embryonic neural tube and neural crest cells (Czeizel & Dudás, 1992;Antony, 2007). Pregnant women require 600ug/day of folic acid, about 5-10 fold that required for non-pregnant woman, for proper fetal development (Li & Rozen, 2006). When these required levels aren't met the fetus takes what it needs from maternal stores. BCRP has been reported to transport conjugated folate and antifolates in membrane vesicles and cell lines (Chen *et al.*, 2003;Volk & Schneider, 2003). Also, folate

deprivation leads to a reduction in BCRP expression and the confinement of BCRP to the cytoplasm of cell line cultures (Ifergan *et al.*, 2004). There have been no studies on BCRP mediated folate transport in human trophoblast cultures.

In conclusion, BCRP is highly expressed by the human placenta and fetal membranes during pregnancy. Through this project we have made significant contributions concerning BCRP expression and its regulation in human gestational tissues. These studies will form basis for the elucidation of the role and function of BCRP in these tissues.

Reference for Final Discussion (page 131-135)

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