EXPRESSION, REGULATION AND FUNCTION OF DRUG TRANSPORTERS IN TISSUES AND CELLS RELEVANT TO HIV-1 SEXUAL TRANSMISSION

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Although some clinical trials have shown the promise of antiretroviral-based topical and oral pre-exposure prophylaxis (PrEP) products in the prevention of HIV-1 sexual transmission, other studies have achieved inconsistent results. Drug exposure in the cervicovaginal tissues, colorectal tissue, and immune cells positively correlates with PrEP effectiveness, and there is an urgent need to identify critical physiologic determinants of drug exposure in these relevant tissues and cells, to inform product optimization. Drug transporters are important regulators of antiretroviral pharmacokinetics. This dissertation aims to examine the expression, regulation, and function of drug transporters in human cervicovaginal and colorectal tissues, as well as in the animal models and cell lines utilized in PrEP product testing.

Multiple efflux and uptake transporters, including P-gp, BCRP, MRP4, 5, 7 and ENT1, were found to be positively expressed in the cervicovaginal and colorectal tissues of humans, macaques, rabbits, and mice. A smaller panel of transporters were positively detected in three epithelial cell lines (End1/E6E7, Ect1/E6E7, VK2/E6E7) derived from human endocervix, ectocervix, vagina, and a T cell line (PM1). Menstrual cycle, exogenous hormones, contraceptives, and proinflammatory cytokines were found to impact transporter expression in mice and/or cell lines. The protein expression of P-gp, BCRP, and MRP4 was demonstrated

using immunohistochemical staining, in the cervicovaginal and colorectal tissues of humans, macaques and mice. The transporters were found to localize at multiple cell types along the cervicovaginal tract, and the protein abundance and localization of transporters were affected by menstrual cycle and hormone/contraceptive use in a mouse model. In a Depo-Provera synchronized mouse model, the co-administration of MRP4 inhibitor MK571 with TFV vaginal gel or intraperitoneal TFV solution significantly increased TFV concentration, in tissues and fluids relevant to HIV transmission. However, MK571 exerted differential effects on the distribution of vaginally administered TFV, when MK571 was given via different routes. Collectively, these studies have shown that antiretroviral drug-related transporters are positively expressed in tissues and cells relevant to HIV-1 sexual transmission, and their expression can be regulated by hormones and cytokines. Further, the studies have provided proof of concept on Mrp4 function in TFV exposure in cervicovaginal and colorectal tissues.

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ABC transporters, ATP-binding cassette transporters; **AhR**, aryl hydrocarbon receptor; **AIDS**, acquired immune deficiency syndrome; **ANOVA**: Analysis of variance; **AR**, androgen receptor; **BCRP**, breast cancer resistance protein; **BV**, bacterial vaginosis; **CAR**, constitutive androstane receptor; **cDNA**, complementary DNA; **CNT**, concentrative nucleoside transporters; **CPM**, count per minute; **CVL**, cervicovaginal lavage; **DEPC**, diethyl pyrocarbonate; **DNase,** deoxyribonuclease; **DPM**, decay per minute; **EI**, entry inhibitor; **ENT**, equilibrative nucleoside transporter; **ER**, estrogen receptor; **FDA**, Food and Drug Administration; **FGT**, female genital tract; **FTC**, emtricitabine; **GAPDH**, glyceraldehyde 3-phosphate dehydrogenase; **GR**, glucocorticoid receptor; **GRAS**, generally-regarded-as-safe; **H & E staining**, hematoxylin & eosin staining; **HIV**, human immunodeficiency virus; **IHC**, immunohistochemical; **II**, integrase inhibitor; **IP**, intraperitoneal; **IL**, interleukin; **IV**, intravenous; **IVC**, inferior vena cava; **LFGT**, lower female genital tract; **MPA**, medroxyprogesterone acetate; **MR**, mineralocorticoid receptor; **MRP**, multidrug resistance associated protein; **MVC**, maraviroc; **N-9**, Nonoxynol-9; **NBF**, neutral buffered formalin; **NNRTI**, non-nucleoside/non-nucleotide reverse transcriptase inhibitor; **NR**, Nuclear receptor; **Nrf2**, nuclear factor (erythroid-derived 2)-like 2; **NRTI**, nucleoside/nucleotide reverse transcriptase inhibitor; **NSAID**, Non-steroidal anti-inflammatory drug; **OAT**, organic anion transporters; **OATP**, organic anion-transporting polypeptide; **OCT**, organic cation transporter; $\boldsymbol{P_{app}}$, permeability coefficient; **PBMCs**, peripheral blood mononuclear cells; **PBST**, phosphate buffered saline solution containing Tween 20; **PD**, pharmacodynamics; **P-gp**, P-glycoprotein; **PK**, pharmacokinetics; **PI**, protease inhibitor; **PMSG**, pregnant mare's serum gonadotropin; **PPAR**, peroxisome proliferator-activated receptor; **PR**, progesterone receptor; **PrEP**, pre-exposure prophylaxis; **PXR**, pregnane X receptor; **RAR**, retinoic acid receptor; **ROR**, RAR-related

orphan receptor; **RT-PCR**, reverse transcriptional polymerase chain reaction; **RXR**, retinoid X receptor; **SC**, subcutaneously; **SLC** transporters, solute carrier transporters; **SIV**, simian immunodeficiency virus; **SHIV**, simian/human immunodeficiency virus; **TDF**, tenofovir disoproxil fumarate; **TFV**, tenofovir; **VDR**, vitamin D receptor.

PREFACE

Finishing this dissertation and my PhD study will not be possible without the great help and guidance from many people. Dr. Lisa Cencia Rohan is the first person I want to express my deep appreciation to, not only because she is my advisor. This work was initiated based on Dr. Rohan's insight and vision of the fields of HIV-1 prevention and drug delivery, and I would not have been able to advance without her guidance. It is my great honor to have a chance to work with her in the past three years.

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1 INTRODUCTION

1.1 HIV global pandemic calls for novel strategies to prevent HIV sexual transmission

1.1.1 Preventing HIV new infections to curtail its global pandemic

Human immunodeficiency virus (HIV) pandemic continues to be a worldwide public health problem, and it is necessary to prevent new infections in order to curtail its pandemic.[1] HIV infection causes acquired immunodeficiency syndrome (AIDS) if not properly treated. AIDS is featured by progressive failure of immune system, which ultimately allows the development of life-threatening opportunistic infections and cancers.[2] HIV has two subtypes: HIV-1 and HIV-2. HIV-1 is the cause of the majority of HIV infections globally. HIV-2 is much weaker than HIV-1 in virulence and infectivity, and is largely confined in West Africa.[3] In 2012 alone, 1.6 million people died of AIDS globally, and the number of people living with HIV was estimated to be 35.3 (32.2-38.8) million in 2012.[1] Since more and more people are receiving the life-saving antiretroviral therapy, the annual AIDS-related deaths have been significantly reduced in the past decade.[1] However, although current therapies can suppress the AIDS symptoms, slow down the disease progression, and reduce the transmission rate to uninfected individuals, they are not able to eradicate the virus in HIV-positive patients.[4] In addition, more than 2 million people are newly infected each year.[1] Taken together, these resulted in a steady increase in the number of people living with HIV worldwide.[1] In order to further reduce AIDS-related deaths, control the number of HIV-positive patients, and ultimately achieve an AIDS-free world, efforts must be made to develop strategies to eradicate HIV from infected patients, and to prevent or significantly reduce the acquisition of HIV in healthy population.[1] While the promise of HIV-eradicating medications

is still unclear,[4] prevention of new infections has been considered as a very important and practical means to curtail HIV pandemic.[1]

1.1.2 Preventing HIV sexual transmission through vaginal and anal intercourse

Currently a great emphasis has been placed on the prevention of HIV sexual transmission. The causes of new infections differ among regions,[1] but sexual transmission is the cause of a vast majority of new HIV infections in sub-Saharan Africa, where 70% of global new infections occur. HIV sexual transmission can occur via the vaginal as well as anal intercourse. Anal intercourse can be practiced by heterosexual couples, or by homosexual men who have sex with men (MSM). The subpopulations with high risk of HIV infection are more likely to practice anal sex, including sex workers, injection drug users, and serodiscordant heterosexual couples (one partner is HIV-positive while the other partner is negative).[5] Although the prevalence of anal intercourse is much lower compared to vaginal sex, the transmission rate through this route is estimated to be 20 times higher, due to a number of anatomical and physiological reasons.[6,7] Therefore, HIV prevention efforts should target the transmissions via both vaginal and anal routes.

Multiple approaches have been demonstrated to be effective in reducing the acquisition rate of sexually transmitted HIV, but novel strategies are needed for HIV prevention. Existing approaches include the use of condoms, male circumcision, reduction in the number of sex partners, and enhancement of the diagnosis and treatment of sexually transmitted infections (STIs).[1,8,9] However, even with the knowledge of these approaches, HIV continues to spread rapidly in developing countries, due to a number of reasons. People's willingness to use condoms is associated with religious, social, and economic factors.[1] Women sometimes cannot negotiate

condom use with male partners, and the condom use is even lower in anal intercourse compared to vaginal sex.[1] The behavior changes, such as the reduction of sex partners, face complex cultural and social challenges and take long time to exert effect. In addition, the facilities and medical professionals required for the diagnosis and treatment of STIs are not readily accessible in many developing countries where the rate of HIV sexual transmission is alarmingly high. Women especially young women (15-24 years) are disproportionally affected in HIV sexual transmission.[1] In sub-Saharan Africa, women constitute about 60% of all the people living with HIV, and the HIV prevalence in young women is more than twice as high as among young men in the same region.[1] Compared to the male partners, women are anatomically more susceptible to HIV sexual transmission.[1] Homosexual men are another group that needs special considerations. In many developing countries, homosexual men are living with social and legal disadvantages, which worsen their ability to access the necessary medical products and services, and increase their vulnerability to sexually transmitted HIV.[1] Based on these considerations, novel convenient strategies that can be controlled by heterosexual women and homosexual men to protect themselves are urgently needed. Such strategies will have high potential to reduce new HIV infections especially in the sub-Saharan countries where the incidence of sexual transmission has been alarmingly high.[1]

1.2 Understanding critical determinants of drug exposure in transmission-related tissues and cells to enhance the effectiveness of pre-exposure prophylaxis (PrEP)

1.2.1 Topical and oral PrEP are promising strategies for the prevention of HIV sexual transmission, but clinical trials yielded inconsistent efficacy results

Pre-exposure prophylaxis (PrEP) is the use of antiretroviral drugs to prevent HIV infection in

uninfected population, and has shown promise in some clinical studies. Currently, most PrEP products are at different stages of development, and these products may be administered orally or topically (vaginally or rectally). The drugs used in PrEP and treatment-for-prevention studies include entry inhibitors (EIs), nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors (PI), and integrase inhibitors (II), which target different steps of HIV life cycle.[6,10-12] The oral and topical PrEP have been considered promising in the prevention of HIV sexual transmission. Many drug candidates undergoing PrEP testing are marketed drugs approved by the U.S. Food and Drug Administration (FDA) for AIDS treatment, or at least have shown good efficacy and safety in preclinical tests.[12-15] Therefore, the use of these drug products, especially via topical administration, has the potential to result in sufficient drug exposure in the tissues relevant to HIV sexual transmission without causing significant toxicities. In addition, the receptive partners don't have to negotiate the use of these drug products. Rather, these products can be used without the cooperation, consent or even knowledge of the insertive partners. An oral product Truvada, which is a tablet containing two reverse transcriptase inhibitors emtricitabine (FTC) and tenofovir disoproxil fumarate (TDF), has been approved by the U.S. FDA for HIV prevention in high-risk uninfected individuals, such as MSM. The PrEP Initiative study (iPrEX) is a key study demonstrating clinical effectiveness and safety toward approval of this product. In this study, the daily Truvada use significantly reduced HIV acquisition in 2499 homosexual men.[16] The vaginally and rectally applied antiretroviral drug products (microbicides) are undergoing development and there is no approved microbicide product to date. Microbicides can be formulated into various dosage forms including gels, films, rings, suppositories and tablets, to meet the diverse needs of different user groups. Compared to the oral route, vaginal and rectal administration could theoretically result in

better accumulation of administered drugs in the cervicovaginal tissues, colorectal tissue, and immune cells associated with these tissues, which are the primary sites of HIV transmission at initial stage. In addition, topical administration results in lower systemic blood drug levels compared to oral administration, and is unlikely to cause systemic side effects. The effectiveness and safety of topical microbicides have been demonstrated in CAPRISA 004 study. In this Phase 2b trial, the 1% vaginal gel of tenofovir (TFV) reduced HIV acquisition by 39% in uninfected women compared to the placebo.[13]

However, PrEP clinical trials have yielded inconsistent effectiveness results, especially for studies that involved TFV. For example, in the topical microbicide trials, the 1% TFV vaginal gel showed 39% reduction in HIV incidence rate in the CAPRISA 004 trial,[13] however the TFV gel arm was discontinued in the VOICE trial due to futility.[17] Among the oral PrEP trials, the iPrEX study which involved daily oral use of Truvada reduced the HIV acquisition in men who had sex with men (MSM).[18] The same oral regimen showed efficacy in serodiscordant couples in the Partners PrEP and TDF2 studies.[14,19] On the contrary, the same FTC/TDF oral regimen failed to prevent HIV-negative women from HIV transmission in the FEM-PrEP and VOICE trials.[15,20] Even for the oral PrEP trials that showed statistically significant efficacy compared to placebo, the determination of effectiveness was not definitive due to the wide confidence intervals.[12]

1.2.2 Tissue drug exposure positively correlates with PrEP efficacy

While many reasons may account for the suboptimal efficacy, several clinical pharmacokinetic studies have revealed the importance of cervicovaginal or colorectal tissue drug exposure in the effectiveness of PrEP. In CAPRISA 004 trial, the TFV concentration in vaginal fluid was

measured in 353 healthy premenopausal women 24 hours after the vaginal administration of 1% TFV gel. A clear trend was observed that the higher TFV concentration in the vaginal fluid, the lower the HIV acquisition rate in participants.[21] HIV acquisition rate in the low exposure group (vaginal fluid tenofovir concentration < 1000 ng/mL) was similar to that in the placebo group, whereas the rate was 3 times lower in high exposure group (> 1000 ng/mL).[21] In the MTN-006 trial, UC781-exposed human colorectal biopsies were cultured *ex vivo* and challenged with HIV. UC781 efficacy positively correlated with its concentration in tissue, as reflected by a high EC_{90}:EC_{50} ratio.[22] These results suggest that the more drug accumulation in cervicovaginal and colorectal tissues, the higher efficacy in HIV prevention.

The importance of tissue drug exposure can be explained by mechanisms of PrEP drug action, HIV transmission, and mucosal tissue physiology. The majority of PrEP drug candidates must reach and/or enter submucosal immune cells to take effect. These drugs prevent the contact between virus and host immune cells (entry inhibitors), or prevent the viral DNA production (reverse transcriptase inhibitors) and integration (integrase inhibitors) after the viruses enter a tiny amount of host immune cells. For example, both tenofovir and UC781 are reverse transcriptase inhibitors. To effectively prevent HIV infection, these drugs must reach and/or enter the immune cells before significant infection occurs, and stay above effective concentration for the entire time window of viral exposure.

Susceptible HIV target immune cells include those in the female genital tract and colorectal tissue, and in tissue-associated lymph nodes, as well as immune cells circulating in the systemic compartment. The circulating immune cells may migrate to lymph nodes/tissues during the early

stage of infection. The immune cells being initially infected during sexual transmission are those residing in female genital tract or colorectal tissues (Figure 1.1). Studies suggest the prevention strategies should intervene at very early stage of HIV infection. During sexual intercourse, the ejaculated viral particles can cross the mucosal epithelial barrier and infect a small group of target cells (T cells, macrophages, dentritic cells with positive expression of CD4 receptor on cell surface) within or underneath the epithelial layer, in a few hours.[23,24] This "founder population" then undergoes local expansion in the first following week to accumulate sufficient viral particles for systemic dissemination. From the second week the viruses start to disseminate throughout the body and establish a highly active infection which becomes difficult to eradicate.[23,24] Therefore, the prevention modalities should be able to prevent the formation and local expansion of the "founder population" at initial stage of viral exposure. This requires rapid and deep penetration of a sufficient amount of antiretroviral drug molecules into the tissue. This tissue level must be retained until the viral invasion risk is diminished, so that tissue-associated immune cells can be fully protected. Since the *in vivo* effective drug concentration for each drug in mucosal tissues has yet to be determined, it has been suggested that one of the goals of future clinical trials is to achieve the highest tolerable drug exposure in the tissues relevant to HIV sexual transmission.[21]

1.2.3 Sufficient tissue drug exposure is difficult to achieve

However, it is challenging to achieve sufficient drug exposure in the tissues, due to behavioral and physiological reasons. Poor patient adherence observed in the clinical trials of once-daily oral and topical PrEP products renders the drug level insufficient to counteract the virus.[12] In CAPRISA 004 trial which tested the effectiveness and safety of 1% tenofovir vaginal gel, 76% of the 335 participants were categorized into the low-exposure group, and had a 3-fold higher

infection rate compared to the high-exposure group. Poor adherence has been revealed to be associated with low drug exposure in this study.[21] In the VOICE trial which tested both TFV vaginal gel and oral tablets containing TFV, most participants did not use the product once daily as instructed.[25] The PK analysis in the blood samples collected from the VOICE trial participants showed that TFV level was detectable in only 23% of the participants in the TFV gel arm.[26] This indicated that three out of four participants did not use the gel product properly.[26] Similarly, in the FEM-PrEP study which tested the oral Truvada tablet, the drug analysis in blood indicated that no more than 30% of women took the tablet as instructed.[27] To address this adherence issue, sustained release and long-acting dosage forms have been actively pursued, and several products have entered clinical phases of evaluation.[28] For topical microbicides, intravaginal rings have been shown to release the antiretroviral drugs in a sustained manner, are generally safe to be used in the female genital tract, and are well accepted by African women.[12,26,28-31] For oral PrEP, a long-acting injectable product containing raltegravir (integrase inhibitor) has shown good safety and effectiveness results in a non-human primate model.[32]

However, even with the emergence of sustained-release and long-acting products, challenges remain toward achieving sufficient tissue drug exposure in a safe and effective manner. Multiple physiological barriers exist and limit drug penetration into the tissues, for both topically and orally administered drugs. The female genital tract and colorectal tissue are covered with a mucus layer which can entrap topically administered drugs and reduce their contact with epithelial cells. In female genital tract, the vagina and ectocervix are lined with multi-layer squamous epithelial cells (up to 40 layers), which serves as a permeation barrier to topical drug administration (Figure 1.1).[33] The endocervix, uterus, and colorectum are lined with single-layer

columnar epithelial cells (Figure 1.1), but the tight junction expression is more intense in these epithelial layers compared to the ectocervical and vaginal epithelia.[34] Moreover, these single-layer tissue regions are at especially high risk of HIV infection and may require different levels of drug exposure than other tissues, because the single layer epithelium is easily breached during sexual intercourse, and the submucosal compartments of these single-layer regions possess more abundant immune cells susceptible to HIV infection.[7] Therefore, achieving sufficient drug exposure in the tissues with single-layer epithelium is not necessarily easier compared to the tissues with multiple epithelial layers. During the sexual transmission, the viral particles can quickly penetrate into mucosal tissues and reach the immune cells residing in the deep stromal sites within a few hours.[24] Since these immune cells are distributed at different depths throughout the female genital and colorectal tracts, the administered drugs must penetrate deeply and accumulate sufficient concentration in the niches surrounding the tissue-associated immune cells. This is difficult to achieve for both topically and orally administered drugs.

For topically administered PrEP drug candidates, the absorption could be problematic, due to the permeation barriers posed by the mucus layer and the epithelium (tight junctional proteins) of the cervicovaginal and colorectal tracts,[33] as well as the blood and lymphatic drainage systems which serve to extract the drug from the tissue (Figure 1.1). This is especially true for hydrophilic drugs that have low binding affinity to tissue proteins. These drugs generally cannot efficiently penetrate the plasma membrane, and they mainly utilize the intercellular space to permeate. A recent Phase I trial (MTN-013/IPM 026) evaluating ring products containing dapivirine (non-nucleoside reverse transcriptase inhibitor, hydrophobic) and maraviroc (entry inhibitor, hydrophilic) revealed that maraviroc was not absorbed well and did not exert protective effect

compared to dapivirine.[35] In this study, three antiretroviral vaginal rings were tested: a ring containing 25 mg of dapivirine, a ring containing 100 mg of maraviroc, and a combination ring containing 25 mg of dapivirine and 100 mg of maraviroc. The fourth ring, a placebo, contained no active drug. The rings were inserted into the vaginal lumen of healthy women for 28 days. The vaginal fluid, cervical tissue, and blood samples were collected at different time points during the 28-day period for the analyses of drug concentration and HIV-preventive efficacy in an *ex vivo* challenge model. Dapivirine was detected in all three types of samples. Although maraviroc dose was 4 times higher, and can be released efficiently from the ring products into vaginal fluid, it was not detected in blood, and the cervical tissue maraviroc concentration can only be detected in 4 of 24 women using either the maraviroc-only ring or the combination ring. In line with the PK findings, the cervical biopsies taken from women using the dapivirine-only ring and the combination ring were able to counteract HIV infection following the *ex vivo* HIV challenge. However, cervical biopsies from the women using the maraviroc-only ring failed to counteract HIV infection.[35,36] Novel strategies that can increase the tissue penetration of hydrophilic drugs will benefit the development of topical microbicides.

For hydrophobic PrEP drug candidates to be applied topically, such as dapivirine, the challenges toward achieving sufficient *in vivo* concentration still exist. Although the hydrophobic drugs can be absorbed more efficiently compared to the hydrophilic drugs, their distribution within the tissues can be potentially problematic. A recent study using excised human ectocervical tissue showed that film-released dapivirine mostly accumulated around the basal layers of cervicovaginal epithelium infiltrating the upper part of the stroma.[37] The amount of drug reaching the deep stroma was very little.[37] This observation posed questions to the design of

microbicide pharmacokinetic/pharmacodynamics (PK/PD) studies and interpretation of the results, especially at the stages of non-human primate (NHP) testing and early phase clinical trials. In these studies, the effectiveness of microbicide products is primarily examined in the *ex vivo* challenge model. In this model, the cervical or vaginal tissue biopsies are taken from the product-exposed participants (NHPs or humans), and cultured *ex vivo* for several days, followed by the challenge of HIV, and measurement of viral proteins as an indicator of HIV replication. The tissue biopsies taken from placebo-exposed NHP or human subjects are used as negative controls.[35,38,39] Since the biopsies are small pieces of tissue, the drug concentration measured in these samples mainly reflect the drug concentration in the epithelial layers and upper part of the stroma. It does not necessarily reflect the drug concentration in the deeper part of the stroma. In the case of dapivirine, the PK/PD results obtained using the tissue biopsies tend to overestimate the drug's capability of penetrating the tissue and preventing HIV infection *in vivo*. This may be true for other hydrophobic drugs as well, which extensively bind to tissue proteins and don't penetrate efficiently into deep stroma. Therefore, strategies that can increase the tissue penetration of hydrophobic compounds will likely improve their *in vivo* PK/PD in HIV prevention.

The tissue distribution of some orally administered antiretroviral drugs also needs to be enhanced for improved effectiveness. PK studies have demonstrated that antiretroviral drugs differ markedly in their ability to penetrate into mucosal tissues and fluids, after oral dosing.[40-43] In the studies focusing on female genital tract, a general trend can be observed that highly protein bound drugs have lower tissue-to-plasma ratios (tissue penetrating ratio, TPR, usually calculated by dividing the area under the concentration-time curve (AUC) of cervicovaginal tissues or

vaginal fluid to the AUC of plasma), because only the free drug portion not associated with plasma albumin or α1-acid glycoprotein can move into tissues. Protease inhibitors (PIs) have the greatest protein binding affinity (95-99% bound to plasma proteins) and generally possess TPRs less than 0.5.[41] Although a number of nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) are less bound to plasma proteins (<49% bound) and possess TPRs higher than 2,[40] some exceptions exist. The TPRs of several NRTIs, including abacavir, stavudine, and didanosine were found to be 0.08 (0.08-0.13), 0.05 (0-0.12) and 0.21 (0.01-0.4), at steady state after oral dosing.[43] The non-nucleoside reverse transcriptase inhibitors (NNRTIs) have drug-specific penetration profile. Efavirenz is an example with low TPR (0.004) in this class.[43] For integrase inhibitors (II), currently tested raltegravir and dolutegravir possess very different TPRs, probably due to the difference in plasma protein binding.[40] Although raltegravir has cervicovaginal tissue and fluid levels several fold higher than plasma level, dolutegravir has relatively high plasma protein binding (>99%) and its TPR was found to be around 0.07.[40,44] Large inter-individual variability has been observed in the female genital tract drug concentration, for drugs within the same class and across different classes.[42] In addition, the variability in drug exposure in female genital tract is generally greater than that observed in plasma.[41,42] Apparently, a better understanding of the causes underlying the low tissue penetration and inter-individual variability will facilitate the achievement of sufficient drug exposure after oral dosing.

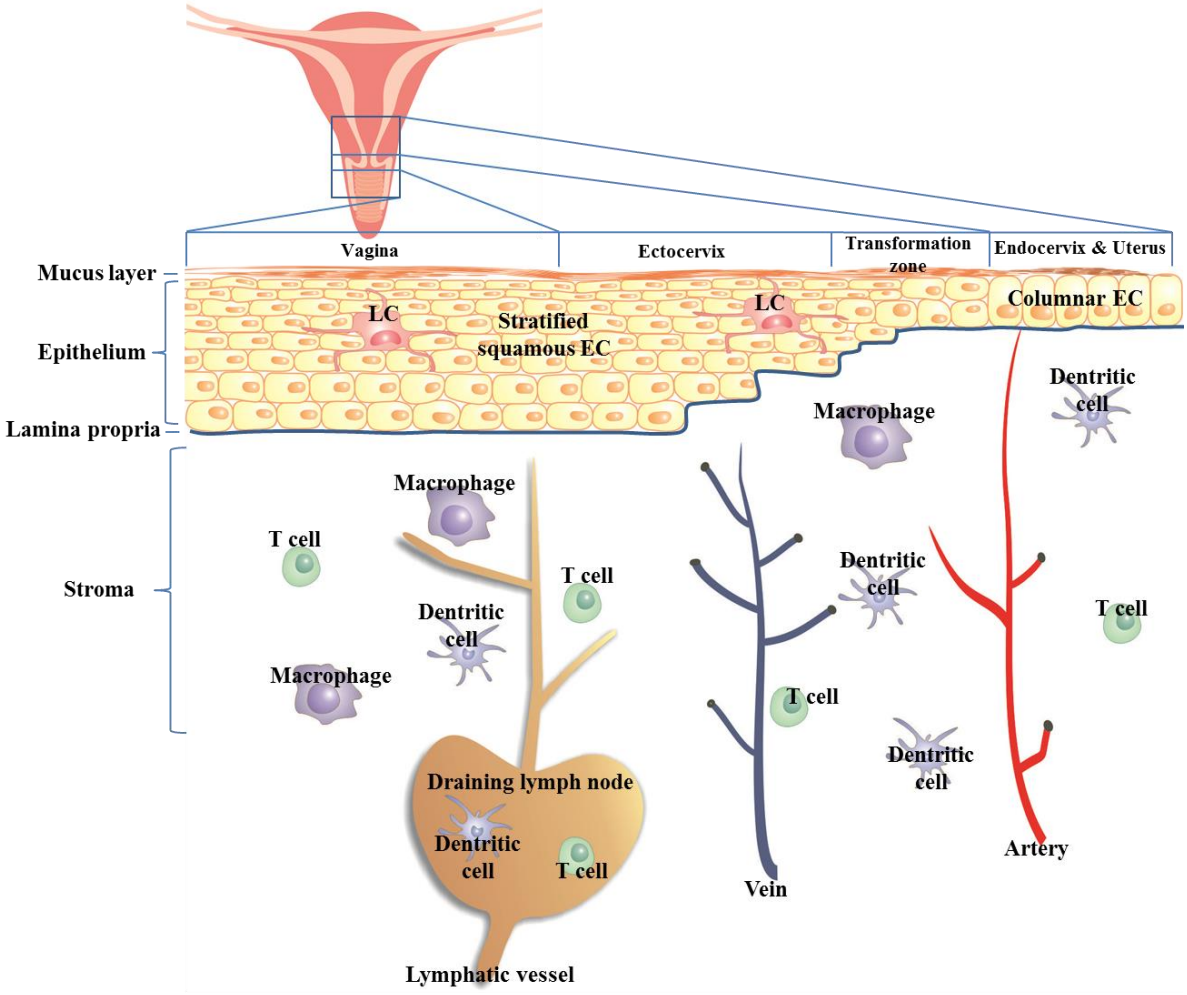


Figure 1.1 Anatomy of the female genital tract.

The female genital tract that can be infected by sexually transmitted HIV include uterus, endocervix, ectocervix, and vagina. A mucus layer covers the epithelia of all these tissue segments, serving as a physical barrier to vaginally administered drugs. Stratified, squamous epithelial layers line the vagina and ectocervix, and a single-layer of columnar epithelial cells lines the endocervix and uterus. The epithelial layers and stroma are separated by the collagen-rich laminar propria. The CD4+ T cells, dentritic cells and macrophages are HIV target cells, and they are distributed in epithelial layers, stroma, and draining lymph nodes. The invading HIV-1 particles can infect these immune cells and establish local tissue infection, expansion, and progress to systemic dissemination. Blood vessels (veins, arteries) and lymphatic vessels mediate the distribution of drug between the tissues and systemic compartments (circulating blood and lymph). LCs, Langerhan's cells, which are the dentritic cells residing in peripheral tissues. EC, epithelial cells. The picture was drawn using CorelDRAW software.

1.2.4 Understanding critical determinants of tissue drug exposure to enhance PrEP drug exposure and efficacy

Currently, the effective *in vivo* drug concentration remains unknown for many microbicide drug candidates being evaluated.[21] Current consensus on PrEP drug delivery is to achieve the maximally tolerated drug concentration in the tissues and cells relevant to HIV sexual transmission.[21] For the antiretroviral drugs with poor ability to penetrate tissues, an easy way of increasing the cervicovaginal tissue drug exposure is to increase the dose. However, high topical dose of some drugs is associated with altered vaginal microbiome and/or genital tract irritation,[45] and poses challenges to the manufacturability and applicability of microbicide products given limited drug loading capacity of topical products. For the oral PrEP, high doses of antiretroviral drugs may cause a variety of toxicities to liver, kidney, and cardiovascular system.[46]

The necessity and challenges of achieving high drug exposure in a safe and effective manner call for a need to better understand critical determinants of antiretroviral drug exposure in the tissues and cells relevant to HIV-1 sexual transmission. An enhanced understanding of tissue exposure determinants could benefit PrEP optimization, in several aspects. First, the new knowledge will enable better understanding of the causes of intra-individual and inter-individual variability in antiretroviral drug PK/PD, and will inform clinical trial design and data interpretation toward individualized regimens. Secondly, the new knowledge will facilitate the development of novel strategies that enhance tissue drug exposure. This will not only benefit drug candidates tested toward PrEP application, but will also help antiretroviral drugs used in AIDS treatment. Since the increased drug exposure in the cervicovaginal tissues and fluids presumably will result in lower viral load in these compartments of HIV-infected women, the improvement of tissue exposure of

antiretroviral drugs will likely reduce the risk of female-to-male sexual transmission, and will ultimately facilitate PrEP in both receptive and insertive partners, and help reduce the number of HIV-positive patients. The enhanced tissue exposure of drugs used in PrEP and treatment will in turn enlarge the pool of drug candidates that can be selected for PrEP testing, and will reduce the drug dose to be administered and minimize the risk of toxicity associated with high dose. To summarize, further understanding of critical determinants of tissue drug exposure is necessary for PrEP product optimization.

1.3 Drug transporters play an important role in antiretroviral drug pharmacokinetics

1.3.1 Classification and transport mechanisms of efflux and uptake transporters

Drug transporters are transmembrane proteins that control the movement of substrates in and out of the cell, and they localize on plasma membrane or the membrane of intracellular organelles such as mitochondria.[47] Numerous studies have established the role of transporters in controlling drug accumulation, and in maintaining the homeostasis of endogenous substances in multiple tissues/organs including brain, liver and kidney,[47-56] and in various types of cells including epithelial cells, endothelial cells and immune cells. ATP-binding cassette (ABC) and solute carrier (SLC) superfamilies are transporters that are most relevant to antiretroviral drugs. ABC transporters are mainly for efflux function. They contain a transmembrane domain which allows the flow-through of substrates, and an ATP binding cassette which binds the intracellular ATP molecules and facilitates their hydrolysis (Figure 1.2A). Using the energy released from ATP hydrolysis, the transmembrane domain undergoes conformational change and pumps the intracellular substrates to the extracellular space (Figure 1.2A).

SLC transporters are mainly for uptake. They are localized on plasma membrane or the membrane of intracellular organelles such as mitochondria. The transport mechanisms of SLC transporters include facilitative transport and secondary active transport. The facilitative transport allows the substrates to flow with concentration gradients (Figure 1.2B). The secondary active transport allows the substrate to flow against the electrochemical gradient, and couple this process to the transport of another substance with its gradient, so that favorable overall free energy change can still be achieved (Figure 1.2C).

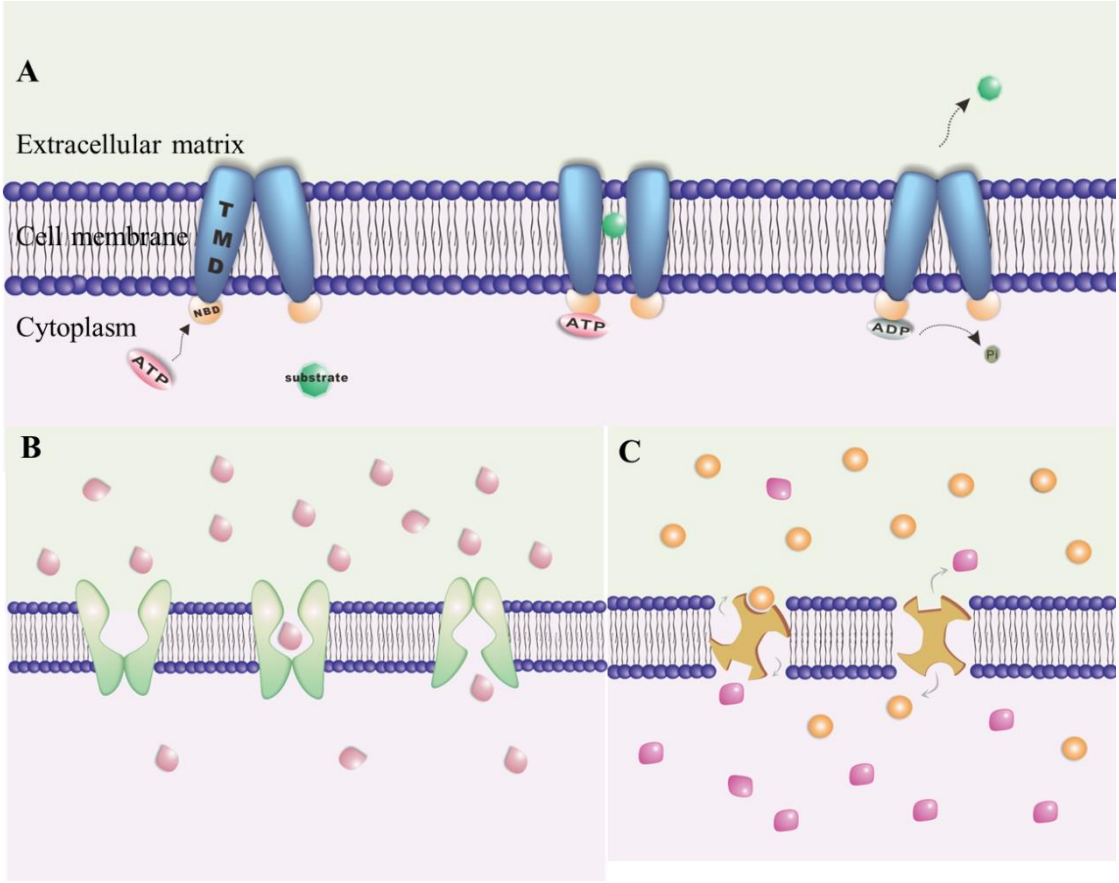


Figure 1.2 Transport mechanisms of ABC and SLC transporters.
A, ABC transporters. NBD, nucleotide binding domain, TMD, transmembrane domain. B, SLC transporter (facilitative transport). C, SLC transporter (secondary active transport). Purple particles, first substrate. Yellow particles, secondary substrate. Pictures were drawn using CorelDRAW software.

1.3.2 ABC transporters in the pharmacokinetics of antiretroviral drugs

In humans, 49 genes have been identified so far in the ABC transporter superfamily. These transporters are further categorized into 7 families, from ABCA to ABCG.[57,58] In this chapter, emphasis is given to ABCB1 (P-glycoprotein) from the ABCB subfamily, ABCC1 to 7 (multidrug resistance associated protein 1 to 7) from the ABCC subfamily, and ABCG2 (breast cancer resistance protein) from the ABCG subfamily, since they comprise the major efflux pumps for antiretroviral drugs.

ABC transporters affect the intracellular accumulation of all classes of antiretroviral drugs, such as maraviroc (EI), saquinavir (PI), tenofovir (NRTI), efavirenz (NNRTI), and raltegravir (II). In addition to xenobiotics, ABC transporters also could affect the homeostasis of endogenous substances, and participate in physiologic processes, such as the immune response. Apart from being the substrates, xenobiotic and endogenous substances could also inhibit the transporter activity by competitively binding to the critical domains for transporter activity. For example, the protease inhibitors (e.g. ritonavir) are potent P-gp inhibitors, which exert the inhibitory effect through competitive binding to the extracellular domain of P-gp.[47,49] Besides marketed drugs, some generally-regarded-as-safe (GRAS) excipients have been shown to potently inhibit ABC transporters, by temporarily depleting intracellular ATP availability and/or reversibly modifying plasma membrane fluidity.[47,59]

In tissues with high expression and/or critical localization of transporters, differential tissue distribution patterns of the substrates vs. non-substrates have been observed. In addition, genetic polymorphisms of a number of transporters are associated with inter-individual variability in

antiretroviral drug pharmacokinetics (PK). Up-regulation or down-regulation of transporter expression/activity could alter antiretroviral drug PK profile and efficacy. Co-administration of efflux transporter inhibitors, including antiretroviral drugs and pharmaceutical excipients, has been shown to be an effective approach to enhance drug exposure in tissues with high level of transporter expression. Since transporter expression and activity can be modulated by many drugs, a significant portion of drug-drug interactions is mediated by transporters. More detailed information on this topic can be found in published reviews.[47,48,60]

1.3.2.1 ABCB1 (P-gp)

Many antiretroviral drugs are substrates of P-gp.[49] All protease inhibitors (PIs) are high-affinity substrates of P-gp.[61-66] In addition, the entry inhibitor maraviroc, the NRTIs such as tenofovir disoproxil fumarate (TDF) and abacavir, and some integrase inhibitors such as raltegravir, can be transported by P-gp. P-gp belongs to the B family (ABCB1) of ABC superfamily. P-gp is the first characterized ABC transporter, and is probably the most extensively studied efflux transporter. It is widely distributed in a variety of tissues including liver, kidney, intestine, brain and immune system. At cellular level, P-gp is localized in epithelial cells, vascular endothelial cells, as well as the immune system cells. Therefore, this transporter could affect drug transport across physiologic barriers that are constructed by epithelial cells (e.g. small intestinal enterocytes) and endothelial cells (blood-brain barrier), and in the immune cells. P-gp has been shown to possess up to five binding sites,[67] which confer broad substrate specificity. P-gp substrates possess diverse structures, but most substrates are hydrophobic, with a molecular weight ranging from 200-300 Da.[68] Due to the wide tissue distribution and broad substrate specificity, P-gp has been recognized as the major efflux pump responsible for cellular resistance to antiviral and anticancer

drugs. The most significant increase of drug accumulation after P-gp inhibition was observed in the brain, as demonstrated in the cell cultures derived from blood-brain barrier, and in mice lacking the P-gp gene or treated with chemical inhibitors against P-gp. Among antiretroviral drugs, some PIs and NNRTIs can inhibit P-gp.[69-74] Moreover, a select panel of pharmaceutical excipients are potent P-gp inhibitors, such as pluronic P85, Vitamin E TPGS, and polysorbate 80.[59] The plasma AUC after oral administration of MVC (P-gp substrate) was 3 times higher in P-gp knockout mice compared to the wild type mice.[75] In humans, the concomitant use of P-gp inhibitors atazanavir, ritonavir, saquinavir and ketoconazole have been shown to significantly increase the C_{max} and AUC of orally administered MVC up to 5 fold.[76] Therefore, P-gp is one of the most important transporters in antiretroviral drug PK/PD. When investigating transporters in HIV transmission-related tissues and cells toward enhancement of drug exposure and effectiveness, P-gp should appear on the priority list.

1.3.2.2 ABCCs (MRPs)

MRPs belong to the C family (ABCCs) of ABC superfamily, and transport a diverse array of antiretroviral drugs.[49] MRP transporters are also widely distributed in various tissues. Similar to P-gp, MRPs also exhibit multiple binding sites for different substrates, and thus are able to transport a wide variety of intact substances, as well as their organic anion conjugates.[59] The MRP members most relevant to antiretroviral drugs are MRP1, 2, 4 and 5. MRP1 and MRP2 are shown to transport several PIs, including ritonavir, lopinavir, atazanavir, saquinavir, and indinavir.[77-81] MRP1, 2 and 4 also mediate the efflux of many NRTIs. For example, MRP1 mediates emtricitabine efflux from lymphocytes. MRP2, 4 and 5 transport tenofovir.[82-85] Administration of TFV to Mrp4 knockout mice resulted in more than 2-fold higher TFV

concentration in the kidney after intravenous administration of TFV.[84] In humans, genetic ABCC4 3463G variants lead to TFV-DP concentrations in peripheral blood mononuclear cells (PBMCs) which was significantly higher than that of wild type.[86] MRP5 has been shown to transport stavudine in vitro.[83] NNRTIs and NRTIs including delavirdine, efavirenz, and emtricitabine inhibit several MRPs at clinically relevant concentrations, as revealed in cell culture experiments.[87] In addition, NSAIDs such as indomethacin and ibuprofen are reported to be potent inhibitors of MRP4,[88-91] and have been demonstrated to enhance the antiviral efficacy of NRTIs in HIV-1-infected T-lymphocytes.[89] Moreover, some excipients are reported to inhibit MRPs, such as pluronic P85, polyethylene glycol (PEG) 300, and cyclodextrins (CDs).[59] Due to the role of MRP transporters in antiretroviral PK/PD, these transporters are important considerations for the enhancement of drug exposure and efficacy in the tissues and cells relevant to HIV sexual transmission.

1.3.2.3 ABCG2 (BCRP)

BCRP belongs to the G family (ABCG2) of the ABC superfamily, and transports a number of antiretroviral drugs from different classes.[49] This transporter was initially characterized in breast cancer cells as an efflux pump contributing to the resistance to chemotherapy. It is also widely distributed in normal tissues. As demonstrated in lymphocytes and BCRP-overexpressed cell cultures, this transporter confers resistance to many NRTIs, including abacavir, zidovudine, lamivudine, didanosine, and stavudine.[92-95] In Bcrp-knockout mice, the brain accumulation of abacavir was significantly increased.[92] BCRP genetic polymorphisms exerted an impact on BCRP activity *in vitro*, however no *in vivo* correlation was observed between BCRP polymorphisms and the concentrations of zidovudine- and lamivudine-triphosphates.[96] Although

PIs and NNRTIs are not BCRP substrates,[97] they have been demonstrated to be potent inhibitors of BCRP. In an *in vitro* assay using human embryonic kidney (HEK) cells stably expressing human BCRP protein, the inhibitory effect of ritonavir, saquinavir, and nelfinavir was studied using mitoxantrone as the substrate, and their IC_{50} values were determined to be 19.5 ± 0.8 μM, 19.5 ± 7.6 μM, and 12.5 ± 4.1 μM, respectively.[97] Weiss *et al.* tested the inhibitory effect of PIs and NNRTIs on human BCRP, using MDCKII cells with or without human BCRP overexpression. The rank order of the estimated IC_{50} values was lopinavir (7.66 μM) > nelfinavir (13.5 μM) > delavirdine (18.7 μM) > efavirenz (20.6 μM) > saquinavir (27.4 μM) > atazanavir (69.1 μM) > amprenavir (181 μM).[98] It should be noticed that the *in vivo* free plasma concentration of PIs and NNRTIs are usually at nanomolar or micromolar range, thus the *in vivo* effect of these drugs on BCRP-mediated transport will have to be tested in properly designed clinical studies.[98] However, the gastrointestinal tract drug concentration is often much higher than the plasma concentration, after oral administration of antiretroviral drugs.[98] The BCRP-inhibiting drugs may at least play a role in the absorption process of co-administered drugs that are BCRP substrates, such as a number of NRTIs mentioned above.[98] Therefore, BCRP plays an important role in antiretroviral drug PK/PD, and should be considered when studying the transporters in transmission-related tissues and cells toward improved HIV prevention and treatment.

1.3.3 SLC transporters in the pharmacokinetics of antiretroviral drugs

SLC transporters are localized on plasma membrane or the membrane of intracellular organelles such as mitochondria. SLC transporters are widely distributed in tissues and cells including liver, kidney, intestine, and immune system cells. The SLC transporters comprise 52 functionally

distinct families with over 300 members. This review focuses on the SLC subfamilies most relevant to antiretroviral drugs, which are organic anion and organic cation transporters (OATs aand OCTs), concentrative and equilibrative transporters (CNTs and ENTs), and organic anion-transporting polypeptides (OATPs), as listed below.

1.3.3.1 SLC22A family members (OATs and OCTs)

OATs and OCTs belong to the SLC22 family. They transport the ionized drugs across plasma membranes. In the pharmacokinetics of antiretroviral drugs, the most extensively studied OATs are OAT1 (SLC22A6) and OAT3 (SLC22A8), which can transport tenofovir. On the basolateral membrane of renal proximal tubular epithelial cells, these two OATs facilitate the uptake of tenofovir from blood circulation into the renal epithelial cells, which could be subsequently effluxed by MRP4 into urine. The genetic polymorphisms of renal SLC22A6 associated with tenofovir-induced kidney toxicity. OCT1 and OCT2 have been shown to transport several NRTIs including lamivudine and zalcitabine.[97] Among antiretroviral drugs, some PIs including indinavir, nelfinavir, ritonavir, and saquinavir, can inhibit OCT1 and OCT2 activities *in vitro*.[98]

1.3.3.2 SLC28 and SLC29 family members (CNTs and ENTs)

CNTs and ENTs belong to the SLC28 and SLC29 families, respectively. They are known to transport nucleoside analogues, including NRTIs. Based on their relative affinities to cellular nucleotides, CNTs are considered as high affinity transporters while ENTs are considered as low affinity transporters.[99] They are ubiquitously distributed in various types of tissues and cells. Among human CNTs, CNT1 and CNT2 mainly transport pyrimidine- and purine-nucleosides, respectively, in a sodium-dependent manner. CNT3 has broad substrate selectivity, and could

transport nucleosides in both sodium- and proton-coupled manner.[100] ENT1 and ENT2 have shown broad substrate selectivity for purine and pyrimidine nucleosides. ENT3 also has broad selectivity for nucleosides and nucleobases, and functions in the membrane of intracellular organelles such as lysosomes.[100] Among antiretroviral drugs, zidovudine and lamivudine are transported by CNT1,[101,102] and didanosine can be transported by CNT2.[103] Zidovudine and didanosine can be transported by ENT3 but the affinity of this transporter was low.[104] The nucleoside reverse transcriptase inhibitors are well-known substrates of ENTs. For example, didanosine can be transported by ENT1 and ENT2, zidovudine can be transported by ENT2,[105] and stavudine and zalcitabine can be transported by ENT3.[60]

1.3.3.3 SLCO family members (OATPs)

The OATPs belong to the SLCO family. OATP1A2 and 1B1 are mostly studied OATPs in antiretroviral drug PK. OATP1A2 and OATP1B1 transport PIs and entry inhibitors including darunavir, lopinavir, saquinavir and maraviroc *in vitro*.[17,106,107] An OATP1B1 polymorphism, 521T>C, was significantly associated with higher plasma levels of lopinavir and maraviroc in patients.[17,107] In addition to being transported by OATPs, some PIs are potent inhibitors of OATP1B1.[108,109]

1.3.4 The effect of antiretroviral drug permeability and solubility on its interaction with transporters

For a given antiretroviral drug, being an *in vitro* substrate of a transporter does not necessarily mean the transporter will play a significant role in the drug's tissue absorption and disposition *in vivo*. The functional role of transporters is affected by the drug's permeability and solubility.[60,110]

Biopharmaceutics Classification System (BCS) categorizes different drugs into 4 classes according to drug permeability and solubility: Class 1, high permeability, high solubility; Class 2, high permeability, low solubility; Class 3, low permeability, high solubility; Class 4, low permeability, low solubility.[110,111] The BCS classification, and the interaction with efflux transporters and uptake transporters are summarized for FDA-approved antiretroviral drugs in Table 1.1 below.

Table 1.1 Summary of the drug transporters that efflux or uptake antiretroviral drugs.

Antiretroviral drug class	Antiretroviral drugs	BCS Class	ABC transporters	SLC transporters
Entry inhibitors (EIs)	Maraviroc	3	P-gp	OATP1B1
Nucleoside/nucleotide reverse transcriptase inhibitor (NRTIs)	Lamivudine	3	BCRP	OCT1, OCT2, CNT1
	Emtricitabine	3	MRP1	
	Tenofovir DF	3	P-gp	
	Tenofovir	3	MRP4, MRP7	OAT1, OAT3
	Abacavir	1	P-gp, BCRP, MRP4	
	Zidovudine	1	BCRP, MRP4	OAT1, OAT2, OAT3, CNT1, CNT3, ENT2
	Didanosine	3	BCRP	CNT2, CNT3, ENT1, ENT2
	Stavudine	1	BCRP, MRP5	CNT1
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	Efavirenz	2	MRP7	
	Nevirapine	2		
	Delavirdine	1		
	Etravirine	4		
Protease inhibitors (PIs)	Atazanavir	2	P-gp, MRP1, MRP2	OATP1A2, OATP1B1
	Ritonavir	2	P-gp, MRP1, MRP2	
	Fosamprenavir	2		
	Amprenavir	2	P-gp	
	Lopinavir	2	P-gp, MRP1, MRP2	
	Saquinavir	2	P-gp, MRP1, MRP2	
	Darunavir	2	P-gp	
	Indinavir	2	P-gp, MRP1, MRP2	
	Tipranavir	2	P-gp	
	Nelfinavir	2	P-gp	
Integrase inhibitors (IIs)	Raltegravir	2	P-gp	

This table is adapted from a review by Kis *et al*.[49] A number of these drugs are already undergoing clinical testing toward the development of PrEP products, including maraviroc, emtricitabine, tenofovir, tenofovir DF, and raltegravir.

For Class 1 drugs, due to their relatively high permeability, they can enter the tissue without the aid of uptake transporters.[60,110] Three NRTIs, zidovudine, abacavir and stavudine belong to Class 1 (Table 1.1). These drugs can easily saturate the efflux transporters because they can quickly enter cells and accumulate high intracellular concentration. Therefore, the Class 1 drugs, including abacavir, zidovudine, stavudine and delavirdine, are not likely to be influenced by efflux and uptake transporters even if they are substrates (Table 1.1).[110]

For Class 2 drugs, they are not likely to be affected by uptake transporters due to high permeability, but they are prone to be influenced by efflux transporters because their low solubility renders them unlikely to saturate efflux transporters as Class 1 drugs can.[60,110] The efflux transporters are able to pump the drug back into the lumen (topical administration) or blood stream (oral administration), and the drug can re-enter the tissue and be repeatedly exposed to intracellular metabolizing enzymes. Therefore, a feature of Class 2 drugs is that they are more likely to be subjected to transporter-enzyme interplay.[110] As shown in Table 1.1, all the PIs, some NNRTIs such as nevirapine, and the integrase inhibitor (II) raltegravir belong to Class 2. Therefore, these drugs will be affected by efflux transporters.

For Class 3 and Class 4 drugs, due to their low permeability, they will require uptake transporters to help them get into tissues.[60,110] The effect of uptake transporters is especially evident for Class 3 drugs which have relatively higher solubility.[60] These two classes also have the potential to be

influenced by efflux transporters, since they are unlikely to achieve high intracellular concentrations that could saturate efflux transporters.[60,110] As shown in Table 1.1, five NRTIs including lamivudine, emtricitabine, Tenofovir DF, tenofovir and didanosine belong to Class 3. The EI maraviroc also belongs to Class 3. Currently only one antiretroviral drug etravirine (NNRTIs) belongs to Class 4. These drugs may be influenced by both efflux and uptake transporters.

To summarize, efflux transporters mainly affect drugs from Classes 2, 3, 4, especially Class 2 drugs. Uptake transporters mainly affect drugs from Classes 3 and 4, especially Class 3 drugs.[60,110] The prediction of the *in vivo* functional role of transporters in tissue drug exposure will need to incorporate the information of drug permeability and solubility, i.e. the BCS classification of antiretroviral drugs, even if the drugs are proven to be transporter substrates *in vitro*.

1.4 Delineating the role of cervicovaginal and colorectal transporters in antiretroviral drug pharmacokinetics

1.4.1 Preliminary evidence suggests the expression and function of drug transporters in transmission-related tissues and cells

A growing body of evidence has demonstrated that some efflux and uptake transporters are expressed in female genital tract, colorectal tissue, and immune cells (summarized below). Studies have demonstrated that efflux transporters of blood-derived immune cells limit the intracellular accumulation and/or efficacy of antiretroviral drugs.[112,113] Although there is little direct evidence showing the functional role of these transporters in drug PK in the tissue, clinical

PK studies comparing drug exposure in different physiologic compartments have implicated the presence of active drug transport mechanisms in the female genital tract.[43,114]

Some transporter substrates, including maraviroc, lamivudine, and emtricitabine, are reported to be preferentially distributed in cervicovaginal tissues and/or fluids at higher levels than in plasma, after oral administration.[43,114] These differential drug distribution profiles cannot be solely explained by the differences in plasma protein binding and passive permeation, as drugs with similar plasma protein binding and permeability displayed different capabilities of distributing into cervicovaginal tissues.[41,43,114,115]

Thompson *et al*. summarized published literature of 58 drugs and developed a mathematical model to predict a compound's cervicovaginal tissue penetration ratio (TPR). In this study, the area under the concentration-time curve (AUC) or single-time-point concentration in cervicovaginal tissues was collected from literature for each drug after oral administration, and was divided by the AUC or single-time-point concentration in the plasma to calculate the TPR. The importance of physicochemical properties, protein binding, and the *in silico* predicted probability of being transporter substrates have been evaluated in the developed model. The analysis showed that the TPR of orally administered drugs was significantly associated with its probability of being the substrate of two efflux transporters MRP1 and MRP4.[116] This analysis highlighted the possibility of *in vivo* functional role of MRP1 and MRP4, in the blood-to-tissue distribution of antiretroviral drugs in lower female genital tract.

1.4.2 The need for further study of transporters in transmission-related tissues and cells to benefit PrEP optimization

A detailed characterization of transporters in the transmission-related tissues and cells will benefit multiple aspects toward PrEP optimization. First, this information is useful in the selection and optimization of PrEP drug candidates. If an efflux transporter is demonstrated to play a significant role in tissue and cell drug exposure, then non-substrates of this transporter may be better candidates than the substrates, if all other attributes of these candidates are similar. On the contrary, if an uptake transporter has a prominent functional role in the tissues and cells, then its substrates may be better candidates than non-substrates for further development. Since the substrate avidity to transporters is determined by chemical structure, the drug candidates could be subject to structural modifications to turn them into non-substrates or substrates, for further development. Secondly, the transporter information can be utilized for the optimization of the microbicide formulation. Chemical modulators, many of which are antiretroviral drugs, could be incorporated into the formulation to inhibit transporter efflux and improve drug penetration and/or tissue retention and efficacy. Alternatively, some pharmaceutical excipients are known to inhibit efflux transporters such as P-gp, BCRP, and MRPs. Finally, transporter information will help explaining and predicting the transporter-mediated drug-drug interaction, and inter-individual variability in antiretroviral PK and PD. An important source for variability is pathophysiological factors including age, gender, and disease status, and the effects of these factors on antiretroviral PK-PD are mediated partially by transporters. Taken together, there is a clear need for more insightful understanding of the transporters in tissues and cells relevant to HIV-1 sexual transmission.

1.5 Existing literature on the expression, localization and functionality of transporters in cervicovaginal tissues, colorectal tract, and immune cells

Table 1.2 Expression and localization of transporters in human tissues and cells relevant to HIV-1 sexual transmission.

Compartments	Tissue/cell type	mRNA/protein expression	Cellular localization	Ref.
Female genital tract	Uterus	P-gp, BCRP, MRP1, MRP4, MRP5, MRP7, OCT3, CNT1, ENT1,2 OATP3A1	Glandular cells of endometrium (P-gp, MRP4, CNT1, ENT1,2); endometrial capillaries (P-gp)	117-122
	Endocervix	P-gp, BCRP	Glandular epithelial cells (P-gp); vascular endothelium of stroma (BCRP)	120,123
	Ectocervix	P-gp, BCRP	Squamous epithelium and stromal tissue (P-gp); vascular endothelium of stroma (BCRP)	119,120,123, 124
	Vagina	N/A	N/A	
Colorectal tissues	Colon	P-gp, BCRP, MRP1, MRP2, MRP3, MRP4, MRP5, MRP7	Columnar epithelium (P-gp, BCRP, MRP3,4,5); secretory epithelial cells (goblet cells) (MRP3,4)	119,122,124-139
	Colorectum	P-gp, MRP2, MRP4	Columnar epithelium and submucosal immune cells (P-gp and MRP2)	136
Immune cells	Within cervicovaginal and colorectal tissues	N/A	N/A	N/A
	Blood-derived	PBMCs: P-gp, BCRP, MRP1,2,3,4,6,7, OCT1, OAT2, ENT1,2, OATP2B1, 3A1		79,86,89,119, 140-154
		Lymphocytes or CD4+ T cells: P-gp, BCRP, MRP1,2, OATP1A2, OATP3A1		113,119,149,155-173
		Macrophages: P-gp, MRP1,4,5,7 CNT1,2,3,		149,174-176

	ENT1,2, OCT1,3, OCTN1,2 Dentritic cells: P-gp, CNT1,2,3, ENT1,2, OCT1,3, OCTN1,2	149,177

1.5.1 Transporters in female genital tract (uterus, endocervix, ectocervix, vagina)

The transporters summarized in Table 1.2 include P-gp, BCRP, MRPs from ABC superfamily, and OATs, OCTs, ENTs, OATPs from SLC superfamily. These transporters have been experimentally demonstrated to transport antiretroviral drugs and are most frequently studied transporters in antiretroviral pharmacokinetics. As listed in Table 1.2, the ABC transporters most relevant to antiretroviral drugs, including P-gp, BCRP, MRP4 are consistently expressed along the entire female genital tract. BCRP appeared to the most highly expressed transporter among ABC transporters in female genital tract. Using RT-PCR, the uterine BCRP mRNA level was found to be the highest in uterus among all types of tissues and organs examined.[122] Several MRP isoforms are also expressed at different segments of female genital tract. Compared to the ABC transporters, SLC transporters were generally less studied in female genital tract. The detection methods for transporter mRNA and protein expression included microarray, conventional RT-PCR, qRT-PCR, and IHC staining. In addition, using IHC and immunofluorescence staining, these transporters were found to locate in cell types that may act as gatekeepers to the drug movement among mucosal lumen, mucosal tissues, and systemically circulating blood and lymph. However, the type of cells that harbour transporter proteins varies among different segments of female genital tract (Table 1.2).

There are only a few reports on transporter function in female genital tract. Uterine P-gp and MRP4 functionalities have been studied in murine and cell culture models, but there is no study

examining transporter function in endocervix, ectocervix, and vagina. Schinkel *et al*. found the Mdr1a/1b double knockout resulted in 2.2-fold increase in digoxin accumulation in mouse uterus after intravenous administration.[178] The MRP4 was demonstrated to be responsible for the extracellular PGE2 release from endometrial epithelial cells, and this release could be inhibited by LXA4 through attenuation of MRP4 mRNA and protein.[121]

The regulation of transporter expression and localization in female genital tract was also reported.[47,113] The cellular localization, staining intensity, and percentage of P-gp in uterus varied with the phase of the menstrual cycle. The staining intensity increased from early proliferative to late proliferative endometrial while diminished from early secretory to late secretory phase.[117] P-gp expression parallels that of nuclear progesterone receptor expression in the normal human endometrial cycle and early gestational endometrium. In addition, P-gp expression corresponds to rising plasma and tissue levels of progesterone as well as to morphologic changes in the endometrial glandular epithelium associated with the marked development of the secretory apparatus.[117] In addition to P-gp, the MRP4 protein expression was found to undergo cyclic change during the estrous cycle in intact bovine endometrial tissues.[179] In the uteri from peritoneal endometriosis patients, Gori *et al*. reported that MRP4 was expressed in eutopic and ectopic endometrium, where it was overexpressed in peritoneal lesions and localized in the cytoplasm of glandular epithelial cells. LXA4 attenuated MRP4 mRNA and protein levels in endometriotic epithelial cells in a dose-dependent manner. This regulation was found to be mediated through estrogen receptor α, as examined using receptor antagonists and small interfering RNA.[121]

1.5.2 Transporters in colorectal tissues (Colon, colorectum)

As shown in Table 1, P-gp, BCRP, MRPs1-7 are positively expressed in human colorectal tissues at moderate to high levels, compared to other genes expressed in colorectum,[119,125,133, 110, 130,136,138] or compared to the expression level of the same transporter in liver.[122] The detection methods include qRT-PCR, Western blot, and IHC staining. De Rosa *et al*. compared the expression levels of multiple ABC transporters in the sigmoid colon of HIV-uninfected man.[110] In this evaluation, the ranking of transporter mRNA level was: MRP2 > MRP4 > MRP1 > P-gp > BCRP, and the ranking of transporter protein level was: MRP1 > MRP4 = BCRP > MRP2 > P-gp.[110] However, Zimmermann *et al*. reported that the rank order of the transporter mRNA level was: MRP3 >> MDR1 > MRP4 almost equal to MRP5 > MRP1 >> MRP2.[138] One possible cause for this difference is the source of tissue samples. De Rosa *et al*. used tissues from young men with an median age of 39, while Zimmermann *et al*. used the tissues from both men and women with the average age of 62.[138, 110] Colorectal P-gp, BCRP, MRP2, MRP3, MRP4 were localized on the plasma membrane of multiple cell types, including specialized epithelial cells with secretory/excretory functions, and endothelial cells of capillary blood vessels.[129,134, 97, 124] Furthermore, MRP4 was localized in endoplasmic-Golgi complex and basolateral location in goblet cells,[134] MRP3 and MRP5 were localized on the basolateral membrane of glandular epithelial cells.[134,180] The regional distribution of transporters along the intestinal tract has also been studied. Blokzijl *et al*. reported that P-gp mRNA level in colon was 5-fold lower than the level in terminal ileum.[125] Prime-Chapman *et al*. examined the mRNA expression of MRP1-6 in human colon and rectum. All the 6 MRPs were detected in the colon, but MRP2 and MRP6 were not detectable in the rectum.[136] As to the SLC transporters, OAT2, CNT1, ENT1 were found to be highly expressed,[119] while OAT1, OAT3, OCT2, OCT3 and OATP1B1 were negligible in

human colorectum.[133]

The functionalities of colorectal P-gp and BCRP have been studied using human tissues and preclinical models. Collett *et al*. studied the permeability of the P-gp substrate UK-338,003 (UK) through mouse ileum and colon. The colonic permeability of UK was at least 40 times lower than that of ileum, with large asymmetry (basolateral-to-apical permeability was much larger than the apical-to-basolateral permeability). The application of P-gp inhibitor markedly increased the colonic permeability of UK to the level comparable to that in ileum. Low apical-to-basolateral permeability of UK was also observed in the *ex vivo* human distal intestine, however the increase in UK permeability after the co-administration of P-gp inhibitor was significantly lower than the increase in mouse colon.[128] Enokizono *et al*. used 4-methylum-belliferone (4MU) as substrate to examine Bcrp functionality on the everted mouse colon sacs. A marked reduction in the mucosal secretion clearance of the 4MU sulfate was observed in the colon of Bcrp knockout mice compared to that of the wild-type mice.[131]

The studies examining the regulation of colorectal transporters have focused on several ABC transporters. The effect of gender on human colonic transporter expression remains unknown, but there is no difference in colonic P-gp mRNA level between male and female mice.[181] The impact of inflammation on transporter expression is different for different transporters. Ulcerative colitis (UC), Crohn's disease (CD), collagenous colitis and diverticulitis cause tissue inflammation and strongly decreased P-gp mRNA and protein expression in colonic epithelial cells and submucosal mononuclear cells. The inflammation also down-regulates BCRP expression.[130] The down-regulation of P-gp is independent of PXR protein level,[125] while the

decrease in BCRP level was negatively correlated with the IL-6 mRNA level.[130] On the contrary, UC and CD significantly increased MRP1 mRNA expression.[125] HIV-1 infection significantly down-regulated P-gp and MRP2 protein levels in male recto-sigmoid colon, while antiretroviral treatment up-regulated P-gp and MRP2 protein levels.[139] The HIV-1 infection and treatment did not have obvious effect on the expression of MRP1, MRP4 and BCRP.[139] In contrast to ABC transporters, the regulation of SLC transporters by infection or antiretroviral treatment is not well understood. Kleberg *et al*. reported that neoplasia up-regulated OATP2B1 and OATP4A1 mRNA levels in human colorectum.[134]

1.5.3 Transporters in HIV-1 target immune cells

Due to the scarcity of human cervicovaginal and colorectal tissues, and the difficulty of purifying the immune cells from these tissues, there is a lack of characterization of the transporter expression and activity of the tissue-associated submucosal immune cells. However, there have been abundant reports on PBMCs, PBMC-derived immune cell subsets, and immortalized immune cell lines, due to the easiness of obtaining the blood-derived immune cells and the experimental convenience of cell manipulation. Numerous studies have revealed the expression profile, functionality, and regulating factors of a number of ABC and SLC transporters in PBMCs, blood-derived lymphocytes or CD4+ T cells, blood monocyte-derived macrophages (MDM), and blood-derived dentritic cells (DCs). The detection methods include RT-PCR, Western blot, and flow cytometry. Large inter-individual differences in P-gp level has been observed in primary lymphocytes isolated from healthy volunteers.[155]

ABC transporters in human blood-derived immune cells have been demonstrated to efflux antiretrovirals and other drugs. In healthy PBMCs, Janneh *et al*. reported the inhibitors of P-gp, MRP1 and MRP2 significantly increased the intracellular accumulation of saquinavir in healthy PBMCs.[79] Janneh *et al*. demonstrated that the MRP inhibitors tariquidar, MK571, frusemide and dipyridamole, as well as protease inhibitors ritonavir, amprenavir and atazanavir significantly increased the intracellular concentration of lopinavir.[158] In lymphocytes, Gupta *et al*. revealed positive efflux activity of P-gp in healthy CD4+ T cells using flow cytometry (Rho123).[157] Clemente *et al*. revealed that the non-steroidal anti-inflammatory drugs (NSAIDs) blocked MRP4 efflux, increased the intracellular concentration and anti-viral efficacy of several nucleoside reverse transcriptase inhibitors (NRTIs) including AZT, in peripheral blood lymphocytes (PBLs).[89] Liptrott *et al*. revealed the nevirapine concentration in the healthy CD4+ blood cells can be increased by small interfering RNA knockdown of MRP7.[170] Lee *et al*. detected MRP1 efflux activity in the CD4+ T cells isolated from healthy subjects, using flow cytometry.[182] The lymphocyte transporter has also been studied in mouse. Elliott *et al*. revealed the positive expression and activity of P-gp (Mdr1a/1b), Mrp1, and Bcrp in mouse blood lymphocytes, using RT-PCR and flow cytometry.[164] Schinkel *et al*. examined P-gp function in mice, and found that the lymph node accumulation of IV administered digoxin was significantly increased in Mdr1a/1b double knockout mice, compared to the wild type mice.[183] In human monocyte-derived macrophages (MDMs), the P-gp inhibitor PSC833 and the MRP1 inhibitor probenecid increased the intracellular accumulation of AZT in HIV-infected MDM, and increased the efficacy of AZT and indinavir.[175] Liptrott *et al*. reported the functionality of MRP7 in effluxing nevirapine from MDMs, using small interfering RNA that specifically knocked down MRP7.[170] Due to the observed efflux activity of immune cell transporters, the genetic

polymorphisms of P-gp, MRP1, MRP4, MRP5 transporters have been shown to correlate with varying intracellular concentration and/or efficacy of substrate drugs including protease inhibitors and nucleotide reverse transcriptase inhibitors, in PBMCs and blood CD4+ T cells.[86,89,154,171]

Apart from the role in drug efflux, immune cell transporters, especially P-gp, have been suggested to play a role in immune cell physiology and HIV-1 infection process. Since the HIV entry into immune cells involves an interaction of the viral gp41 peptide and the target cell plasma membrane, and that P-gp could transport hydrophobic drugs and peptides, the observed effect of P-gp overexpression on HIV infectivity may be related to its interaction with the critical receptors/co-receptors on immune cell surface.[169] Some researchers reported inverse correlation between lymphocyte P-gp level and HIV infectivity. Lee *et al*. reported P-gp overexpression greatly reduced the HIV infectivity, during the fusion of virus and host plasma membranes, and at later steps of the HIV life cycle, in CD4+ human T-leukemic cell line (12D7). This observed reduction was not due to altered expression of the CD4 receptor or CXCR4 coreceptor on the target cell surface.[169] Speck *et al*. observed the overexpression of P-gp in CEM cells significantly decreased (by at least 70-fold) the HIV protein and infectious virus production, and this effect could be partially reversed by P-gp inhibitors.[173] Hulgan *et al*. found that P-gp activity in CD4+ T cells from 185 HIV-infected patients receiving antiretroviral therapy was inversely correlated with plasma HIV-1 RNA level.[167] Sankatsing *et al*. found a reduced intracellular HIV load in the patients with high cellular P-gp activity, among both therapy-naive and in PI-treated patients.[172] However, there have been controversial reports on the role of P-gp in HIV-1 infection and disease progression. In addition to the studies that suggested the role P-gp in HIV infectivity,

several studies reported no correlation between P-gp level and HIV-1 infection/progression. Bleiber *et al*. revealed that the difference in physiological levels of P-gp did not alter the permissiveness of healthy human CD4+ T cell to HIV *in vitro*. In addition, the P-gp alleles (exons 21 and 26) and haplotypes did not exhibit significant impact on the disease progression before the initiation of antiviral treatment, in the HIV-infected patients.[162] Agrati *et al*. detected no difference in P-gp expression in total PBMC and CD4+ T cells from 3 groups of HIV-infected patients: treatment naïve, responders, and non-responders. This suggested the immune cell P-gp was unlikely to be a determinant of patient response to antiretroviral therapy when P-gp substrates constitute a significant portion of the regimen.[140] Similarly, Bossi *et al*. did not observe an association between CD4+ cell P-gp level and the treatment outcome, in the infected patients treated with protease inhibitors.[163] In addition to P-gp, MRP1 overexpression was also shown to increase viral production in the CEM cells, and this effect can be partially abrogated by MRP inhibitors.[173] In CD4+ T cells, the exposure to HIV-binding glycoprotein gp120 *in vitro* resulted in rearrangement of P-gp protein localization, suggesting an interaction between viral protein and P-gp exists.[147] In addition, P-gp was preferentially associated with glycolipid-enriched membrane (GEM) domains, while MRP1 was not. Since the GEM domain may be an important site for the binding and egress of HIV, the difference between P-gp and MRP1 in GEM association likely resulted in differential effect of the two transporters on the HIV infection process.[173] In addition to the role in HIV-1 infectivity, P-gp was shown to transport a number of endogenous immune mediators including monensin, retinol, IL-2 and IFN-γ, [150] promote lymphocyte survival,[142] and facilitate DC migration into lymphatic vessels.[177]

The effects of disease, drug treatment and activation status on immune cell transporter

expression and activity have been evaluated. The effect of HIV-1 infection depended on the model and cell type selected for examination. In human PBMCs, HIV infection up-regulated mRNA levels of P-gp and MRPs1, 4, 5.[154] In H9 (T cell line) and U937 (monocytic cell line) cells, HIV infection increased the expression of P-gp and increased the efflux of P-gp substrates AZT and daunorubicin.[165] In MDMs, HIV infection increased the mRNA levels of MRP1, 4, 5.[174] However, Lucia *et al*. reported that viral load did not affect P-gp expression in the PBMCs of treatment-naive and –experienced patients.[145] In cynomolgus macaques, HIV infection decreased P-gp mRNA level in PBMCs and lymph node mononuclear cells.[143] Besides HIV infection, myasthenia gravis decreased P-gp efflux function of PBMCs,[152] and increased intracellular cholesterol levels markedly up-regulated P-gp activity in PBMCs.[153] The effect of cytokine and antiretroviral/anticancer drug treatment on immune cell transporters appeared to be drug-specific. IL-2 and IFN-γ significantly increased the mRNA and protein expression of P-gp, MRP1 and MRP2, and reduced accumulation of digoxin and saquinavir, in the PBMCs of healthy volunteers.[144] In MDMs, IFN-γ increased P-gp mRNA abundance, induced polarized redistribution of P-gp protein in pseudopodia, and increased the efflux activity of P-gp.[176] The anticancer treatment of Kaposi sarcoma (KS) using the liposomal formulation of anthracycline doxorubicin (L-DOX) up-regulated P-gp in the PBMCs of treatment-naive and –experienced patients.[145] AZT increased the MRP4 expression level in HIV-1 infected T lymphocytes.[89] Despite these up-regulating effects, Lucia *et al*. reported HAART did not affect P-gp expression in the PBMCs of treatment-naive and -experienced HIV-infected patients.[145] Agrati *et al*. reported that antiretroviral treatment did not affect P-gp expression in PBMCs of HIV-infected patients.[140] Bossi *et al*. found that the protease inhibitor treatment had no effect on the P-gp expression and activity, and was not linked to the treatment outcome.[163] Chinn *et al*. observed no

significant effect of saquinavir and atazanavir treatments on P-gp expression (qRT-PCR) and activity (Rho123) of lymphocytes isolated from healthy subjects.[155] In addition, Lucia *et al*. found that treatment with protease inhibitors, including ritonavir, saquinavir, neifinavir and indinavir, reduced the efflux of rhodamine 123 in blood lymphocytes from healthy and HIV infected patients.[146] Lee *et al*. reported that protease inhibitors exerted differential effects. MRP1 expression and efflux activity in healthy CD4+ T cells were reduced after the treatment of darunavir/ritonavir, but not after efavirenz treatment alone.[182] In HIV infected cynomolgus macaques, HAART (zidovudine [AZT], lamivudine [3TC], and indinavir [IDV]) accentuated the infection-induced decrease of P-gp mRNA level, in macaque PBMCs and lymph node mononuclear cells.[143] The chemical-induced activation was shown to increase the expression and/or activity of P-gp and ENTs in primary lymphocytes.[149,157]

1.5.4 Summary of existing literature

The positive expression of multiple transporters in the tissues and cells relevant to HIV transmission indicates that these transporters may play a role in drug absorption and disposition and PrEP effectiveness. Especially, some important transporters (P-gp, BCRP, MRPs) have been demonstrated to localize in multiple cell types (epithelial cells, secretory glandular cells, vascular endothelial cells) in these tissues, indicating these transporters may affect multiple processes of drug handling in the tissues and cells. The uptake transporters were generally less studied compared to efflux transporters. OCTs, CNTs, ENTs, and OATPs can be detected in some tissues, however the expression profiles of OAT1 and OAT3, which are the major uptake transporters for tenofovir, remain unknown in the tissues and immune cells. The positively expressed transporters may be able to affect the drug pharmacokinetics in all the three compartments in HIV-1

transmission (mucosal fluid, tissue, and blood). The observation that transporters (e.g. P-gp) can be regulated by exogenous hormones, disease status, and concomitantly used drugs highlighted the possibility that the transporter functionality may be variable under different pathophysiological conditions.

Contradictory reports have been observed for transporter expression and activity. This is especially evident in immune cells. There is large heterogeneity between different subsets of the immune cells, and even for the same subset, for example, $CD4^+$ T cells, there have been contradictory reports regarding transporter expression.[112] However, MRP1 appeared to be consistently reported to express at high level in human lymphocytes, indicating that this transporter may play a consistently significant role in the pharmacokinetics of antiretroviral drugs in the immune cells.

The reasons for these conflicting results may include differences in the experimental approaches, including different methods of detection, different reagents, and different models. For example, for expression analysis, RT-PCR and Western blot may yield different results, as the correlations among mRNA level and protein level are not always good. The antibodies used for the WB, IHC staining and flow cytometry may differ in the sensitivity and/or specificity, and may render different signal intensity for the same sample. When testing the role of transporters in HIV-1 infection, the selection of infection model may have strong impact on the results and conclusion.

In addition to the differences in experimental approaches, the potential inherent heterogeneity in immune cells being tested may also contribute to the observed conflicting results for transporter

expression and activity in immune cells. It has been reported that many factors influence transporter expression and activity (varying level of sex hormones, age, gender, methods of lymphocyte activation) etc.[112] If the blood is obtained from patients under the influence of these factors, then a large heterogeneity in blood cell transporters may be unsurprising.

1.6 Knowledge gaps that preclude direct utilization of existing knowledge on cervicovaginal and colorectal transporters in PrEP optimization

First of all, there is a lack of systematic characterization of the mRNA and protein expression of transporters in human lower genital tract, colorectal tissue, and in the animal models and cell lines used in PrEP drug candidate screening. This information is important for a comprehensive understanding of human tissue transporter expression, and is necessary for the evaluation of the validity of the preclinical models used in the PrEP drug candidate screening. In addition, the characterization of transporter expression in the preclinical models will provide valuable information on the utility of these models in the studies of transporter regulation and function, as discussed below.

The effect of PrEP-relevant factors on transporter expression remains largely unknown. These factors include varying levels of sex steroid hormones (due to menstrual cycle and menopause), exogenously administered hormonal contraceptives, and local tissue inflammation caused by bacterial or fungal vaginosis. These factors have been demonstrated to impact transporter expression and activity in metabolic organs such as liver, but few studies investigated their effect on cervicovaginal and colorectal tissue transporters. A significant number of these regulations are mediated by nuclear receptors (NRs), which are transcriptional factors that regulate mRNA

expression of downstream genes through binding and activating the promoter region. The information generated from these studies will be helpful for the understanding of the dynamics of transporter expression in cervicovaginal and colorectal tissues, and will rationalize the study of transporter function in these tissues, as discussed below.

The role of the positively expressed transporters in PrEP drug pharmacokinetics remains undetermined. It is not clear whether the positive expression of transporters correlate with transport activity, in the cervicovaginal and colorectal tissues. The transporter function in female genital and colorectal tissues has been occasionally studied, as a barrier to topically administered drug intended for systemic absorption. However, for these tissues, it is more important to know whether the transporters could affect drug distribution and retention within the tissues, since the tissue-associated HIV-1 target immune cells reside within the tissues. As discussed above, these functionality studies need to be conducted in a PrEP-relevant setting. The effect of PrEP-relevant factors on the function of transporters in genital tract and colorectal tissue warrants further studies.

1.7 Hypothesis and Specific Aims

Based on the summary and discussion above, emerging evidence has suggested that efflux and uptake transporters are positively expressed in the female genital tract and colorectal tissues, which are the primary sites of HIV-1 sexual transmission. Further study is warranted to systematically examine the expression and localization of transporters in these tissues, especially in female lower genital tract. In addition, it is necessary to examine the effect of PrEP relevant factors (menstrual cycle, exogenous hormones and contraceptives, cytokines implicated in

cervicovaginal tissue inflammation) on transporter expression. Finally, it is crucial to test the function of select transporters in tissue pharmacokinetics of topically and systemically administered antiretroviral drugs. To address these research questions,

We hypothesize that multiple efflux and uptake transporters are positively expressed in cervicovaginal tissues, colorectal tract, and immune cells, and their expression can be regulated by steroid hormones and cytokines. We further hypothesize that positively expressed efflux transporters play a role in tissue drug exposure, which can be reversed by transporter inhibitors.

This hypothesis will be tested in the following three specific aims:

Aim 1 will examine mRNA expression profiles of antiretroviral-relevant efflux and uptake transporters in human cervicovaginal and colorectal tissues. The transporter expression profile in animal and cell models used in PrEP testing will also be examined and compared to the expression patterns in human tissues. In addition, the effect of a panel of PrEP relevant factors on transporter expression will be evaluated using human tissues or preclinical models, including menopause, menstrual cycle, exogenous hormones, contraceptives, and proinflammatory cytokines. The results from Aim 1 will identify highly expressed transporters for further characterization at the levels of protein expression and functionality.

Aim 2 will examine the protein expression of highly expressed transporters in cervicovaginal

tissues of human and animal models used in PrEP product testing, under the influence of PrEP relevant factors listed in Aim 1. The results generated in Aim 2 will confirm the mRNA results in Aim 1, and will provide valuable information for the design and interpretation of transporter function studies.

Aim 3 will test the function of a select transporter in substrate distribution in tissues relevant to HIV-1 sexual transmission. According to the results generated from Aims 1 and 2, one transporter will be selected based on expression level, relevance to PrEP, and availability of research models for this particular transporter. The studies in this aim will provide proof of concept on transporter function in cervicovaginal and/or colorectal tissues, in a model utilized in PrEP product evaluations.

2 RNA EXPRESSION OF TRANSPORTERS IN TISSUES AND CELLS RELEVANT TO HIV-1 SEXUAL TRANSMISSION

2.1 Introduction

As discussed in Chapter 1, antiretroviral drug exposure in the tissues and cells relevant to HIV-1 sexual transmission positively correlates with PrEP efficacy. It has been suggested that one of the goals of future PrEP clinical trials is to achieve maximally tolerable drug concentration in the female lower genital tract. Toward this goal, it is imperative to understand the expression, regulation, and function of drug transporters in cervicovaginal and colorectal tissues, as well as in the HIV-1 target immune cells. The knowledge generated from these studies will facilitate better understanding of differential drug distribution profiles of different antiretroviral drugs, as well as intra-individual and inter-individual variability in mucosal tissue drug pharmacokinetics. In addition, researchers could utilize this information to develop novel strategies to improve the exposure and efficacy of antiretroviral drugs in the mucosal tissues and immune cells.

However, functional investigation of cervicovaginal transporters is hampered by a lack of systematic and detailed characterization of cervicovaginal transporters in both human and preclinical models used in PrEP testing. The transporters' role in antiretroviral pharmacokinetics is complex. A lot of efflux and uptake transporters affect the absorption and disposition of antiretroviral drugs, and one drug may be the substrate of multiple transporters. For example, tenofovir, the most extensively studied drug candidate in topical microbicide development, is the substrate of two efflux transporters (MRP4, MRP7) and two uptake transporters (OAT1, OAT3). The human female lower genital tract is composed of endocervix, ectocervix and vagina, which

are anatomically and physiologically distinct from each other. In addition to female genital tract, the colorectal tissue is an even more vulnerable site of HIV-1 transmission, because it has single layer epithelium that is easily disrupted during sexual intercourse, and the submucosal immune cells are more enriched in colorectal tissue. Therefore, the expression analysis of a comprehensive panel of all the transporters relevant to antiretroviral drugs, in a tissue region-specific manner, is needed prior to the functional characterization of transporter function and modulation in tissues.

The aim of the studies described in this chapter was to examine the mRNA expression of efflux and uptake transporters in the cervicovaginal and colorectal tissues of human and animal models (macaque, rabbit, mouse), as well as in the cell lines derived from human cervicovaginal tissues (End1/E6E7, Ect1/E6E7, VK2/E6E7) and immune system (PM1). Since PrEP participants could encounter factors including contraceptives and local tissue inflammation, and these factors have been reported to affect transporter expression and activity in other tissues, we seek to examine the effect of these factors on transporter expression in transmission-related tissues and cells, using mouse model and cell lines derived from cervicovaginal tissues and immune system. Since nuclear receptors (NRs) are important mediators of transporter regulation, we examined the mRNA expression of 23 transporter-related NRs in human, macaque, and mouse tissues, to explain the observed effect of hormones/contraceptives/cytokines, to provide clues for the prediction of other factors that may regulate cervicovaginal transporters, and to provide a basis for multispecies comparison of transporter regulation mechanisms. The information revealed in this chapter will identify highly expressed transporters in the mucosal tissues and immune cells relevant to HIV-1 sexual transmission, under the conditions that are commonly encountered by

PrEP participants. This will in turn inform the prioritization of future functional characterization of transporters in these tissues and cells.

2.2 Materials and Methods

2.2.1 Procurement of human, macaque, and rabbit tissues

All human tissues (endocervix, ectocervix, vagina, sigmoid colon, liver) were obtained through the Tissue Procurement Facility at University of Pittsburgh Medical Center under IRB approved protocols. The gender and age range of the tissue donors, and tissue usage information were summarized in Appendix. Human ectocervical tissues were obtained from pre- or postmenopausal women undergoing hysterectomy for benign conditions. Human vaginal tissues were collected from premenopausal women with vaginal prolapse. Female pigtailed macaque tissues (endocervix, ectocervix, vagina, colorectum, liver) were obtained from the Washington National Primate Research Center at the University of Washington, through the Tissue Distribution Program. The animals were maintained in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals. The ages of the three macaques used in this study were 12.6, 18.7, 17.6 years. The rabbit tissues were obtained from euthanized New Zealand White rabbits, which were a kind gift from Dr. Eric Romanowski of the Eye & Ear Institute of University of Pittsburgh. The tissue collection was in line with the regulations of the Institutional Animal Care and Use Committee (IACUC) of University of Pittsburgh. The rabbits were 6-8 weeks old at the time of euthanasia. Before euthanasia, the rabbits were used for the testing of topical drug products for the treatment of eye infection. The infection and drug action were contained in the cornea, and these procedures exerted no systemic effect on the tissues collected for transporter study.

2.2.2 Collection of tissues from mice undergoing natural cycling or being synchronized with PMSG/Depo-Provera

All animal procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC). Female Swiss Webster mice (6 weeks old, around 23 g body weight) were used for all mouse experiments. Tissues including uterus, endocervix, ectocervix, vagina, colorectum, and liver were collected under 4 conditions: estrus and diestrus stages in the natural estrous cycle, synchronized estrus stage, and synchronized diestrus stage. For the collection of tissues from mice undergoing natural estrous cycle, mouse estrous cycle stage was examined daily, and tissues were collected from euthanized mice when the estrus or diestrus stage was reached. Mouse estrous cycle stage was determined using vaginal cytology as described by Caligioni *et al.*[184] The mouse vaginal lumen was injected with 20 µL of normal saline. The smear was taken out and evenly spread onto a glass slide and observed under a bright light microscope. The smear from proestrus stage predominantly consists of small nucleated epithelial cells. The smear from estrus stage is featured by the anucleated cornified epithelial cells which are much larger and darker than the cells found in the proestrus. The smear from metestrus stage consists of three types of cells: leukocytes, cornified, and nucleated epithelial cells. The smear from diestrus stage predominantly consisted of leukocytes. For the collection of mouse tissues under synchronization, mice were treated with pregnant mare's serum gonadotropin (PMSG, Sigma) or Depo-Provera (Pfizer Inc.), for the synchronization into estrus or diestrus stages, respectively. For PMSG treatment, mice were intraperitoneally (IP) injected at the dose of 5 IU per mouse (around 23 g). For Depo-Provera treatment, mice were

subcutaneously (SC) injected twice, on Day 1 and Day 5, at the dose of 3 mg per mouse. Synchronized estrus stage was reached 24 hours after PMSG administration, and the synchronized diestrus stage was reached 7 days after the first time injection of Depo-Provera. The estrous cycle stage of the synchronized mice was confirmed as described above, tissues were collected and stored at -80 °C until further analysis. For each of the 4 conditions, tissues were collected from at least 3 mice.

2.2.3 Cell culture and treatment of contraceptives and cytokines

End1/E6E7, Ect1/E6E7, VK2/E6E7, and PM1 cells were cultured as per the protocols recommended by American Type Culture Collection (ATCC), at 37 °C under 5% of carbon dioxide (CO_2). For End1/E6E7, Ect1/E6E7, and VK2/E6E7 cells, the culture medium was keratinocyte-serum free medium (GIBCO-BRL 17005-042) with 0.1 ng/mL human recombinant EGF, 0.05 mg/mL bovine pituitary extract, an additional 44.1 mg/L calcium chloride (final concentration 0.4 mM). Antibiotic combination containing 50 IU/mL penicillin and 50 µg/mL streptomycin was also supplemented into the culture medium to prevent microbial contamination. For PM1 cells, the culture medium was RPMI 1640 with 10% fetal bovine serum and the supplementation of antibiotic combination containing penicillin and streptomycin. The End1/E6E7, Ect1/E6E7, and VK2/E6E7 cells were initially grown in culture flasks. After 60-90% confluence was reached, the cells were detached using trypsin-EDTA, and diluted in the culture medium to reach the target density (5×10^5 cells per well), followed by seeding onto 6-well plates. The PM1 cells were always cultured in flasks. Cell treatment with contraceptives and cytokines was initiated when the End1/E6E7 and VK2/E6E7 reached 60-80% confluency, and when the PM1 cell density reached 1×10^6 cells per mL. The stock solutions for MPA and P4

were prepared using EtOH, and the stock solutions for IL1β and IL8 were prepared using culture medium. To test the effect of contraceptives, the cells were treated with culture media containing MPA (1 μM) or P4 (1 μM) for 48 h. To test the effect of inflammation, the cells were treated with culture media containing IL1β (10 ng/mL) or IL8 (10 ng/mL) for 48 h. It has been reported that immediately after the dosing of Depo-Provera (containing 150 mg MPA) to patients, the initial plasma level was 1.7 ± 0.3 nmol/L. Two weeks later, the level was 6.8 ± 0.8 nmol/L (http://www.medicines.org.uk/emc/medicine/11121). These plasma concentrations corresponded to 1.7 - 6.8 × 10^{-3} μM, and the concentration of MPA in the preliminary testing reported in this chapter was above physiologic concentrations observed in clinical use of Depo-Provera. The concentrations of IL1β and IL8 were determined based on the levels of these two cytokines observed during bacterial vaginosis (BV) in human female reproductive tissues.[185-187] Yudin *et al*. reported that the median cervical level of IL1β was 3.04 (0.01-22) ng/mL, and the median cervical level of IL8 was 54.72 (5-389) ng/mL, in 111 pregnant women with BV.[187] Cauci *et al.* reported that median IL1β level in vaginal fluid was 0.77 (0.30-1.96) ng/mL, and the median IL8 level was 3.37 (2.36-8.20) ng/mL, in 19 BV patients with high neutrophil count in their vaginal fluid.[185] Imseis *et al.* reported the vaginal level of IL1β to be 3.36 ng/mL in BV patients.[186] Based on these reported levels, the test concentration was set at 10 ng/mL for both cytokines. The control cells were treated with vehicles that were used to dissolve the contraceptives or cytokines. The cells were then collected for RNA extraction and real-time RT-PCR analysis of transporter expression.

2.2.4 RNA extraction from tissues and cells and reverse transcription

RNA of all the tissue and cell samples was extracted using TRIZOL (Invitrogen) as per the manufacturer's instructions. For conventional RT-PCR, RNA was extracted from the epithelium and stroma of human ectocervical tissues, which were separated using hand microtome. For real-time RT-PCR, RNA was extracted from whole tissue pieces which contained both epithelium and stroma in the same sample. For endocervix, ectocervix and vagina, 100-200 mg of tissue was used for each sample. For liver and colorectal tissue, 50-100 mg of tissue was used for each sample. The RNA was dissolved in DEPC-treated H_2O, and the RNA quality and quantity were determined using NanoDrop with 2 µL of dissolved RNA samples. For conventional RT-PCR, the extracted RNA was directly used for reverse transcription using SuperScript III First-strand Synthesis Kit (Invitrogen Inc.). For real-time RT-PCR, the extracted RNA was subjected to DNase treatment to remove contaminating genomic DNA, using the Turbo DNase kit (Ambion), followed by reverse transcription using SuperScript III kit. The prepared complementary DNA (cDNA) samples were used immediately in PCR or stored in -20 °C freezer until use.

2.2.5 Conventional RT-PCR of human tissues

Conventional RT-PCR experiments were performed using cDNA samples from human tissues. Each tissue sample was collected from one patient. In RT-PCR of human efflux transporters (Figure 2.1), the ectocervix and vagina samples were collected from 7 and 5 patients, respectively. The epithelium and stroma of each tissue sample were separated using a hand microtome. For ectocervix, the epithelia from 7 patients were mixed to make pooled ectocervical epithelium, and the pooled stroma were made in the same manner. Pooled epithelia and stroma of ectocervix were then analyzed by RT-PCR, and occupied two lanes in the gel image (Figure

2.1). For vagina, the epithelium and stroma of each of the 5 vagina samples were individually analyzed by RT-PCR (Figure 2.1). In RT-PCR of human uptake transporters (Figure 2.2), the epithelia and stroma of each ectocervix or vagina were separated, and then mixed to make pooled ectocervical epithelium, ectocervical stroma, vaginal epithelium, and vaginal stroma, respectively (Figure 2.2). To quantitate the mRNA level, serially diluted liver cDNA was used as a standard, given the abundant expression of the drug transporters and metabolizing enzymes planned for examination in this study in the liver. Human liver tissue samples were obtained from 6 donors with mixed gender, and pooled for RNA extraction and RT-PCR. PCR was performed using the GoGreen Hot-start mastermix (Promega Inc.). The cDNA transcribed from 50 ng of total RNA was used for all the cervicovaginal tissue samples, while the cDNA from 1, 5, 25 ng total RNA were used for liver standards. Human β_2-microglobulin served as the internal control. The PCR programs consist of a denaturation at 95 °C for 4 min, followed by 28 to 36 cycles of 95 °C 30 sec, 60 °C 30 sec, and 72 °C 30 s. The cycle number for each gene was shown in the Figures 2.1 and 2.2. The final extension was set at 72 °C for 3 min. The PCR products were then analyzed using agarose gel electrophoresis, and gel pictures were captured by using the ChemiDoc™ MP imaging system (Biorad Inc.). The information of primer sequences used in conventional PCR were summarized in Table 2.1.

Table 2.1 Primer information for conventional RT-PCR of human efflux and uptake transporters

Common Gene name (Official gene symbol)	GeneBank accession no.	Primer sequence 5' to 3'	Ref.
P-gp (ABCB1)	NM_000927	Forward: CCCATCATTGCAATAGCAGG Reverse: TGTTCAAACTTCTGCTCCTGA	188
BCRP (ABCG2)	NM_004827	Forward: TGGCTGTCATGGCTTCAGTA Reverse: GCCACGTGATTCTTCCACAA	189
MRP1 (ABCC1)	NM_004996	Forward: ATGTCACGTGGAATACCAGC	188

		Reverse: GAAGACTGAACTCCCTTCCT	
MRP2 (ABCC2)	NM_000392	Forward: ACAGAGGCTGGTGGCAACC Reverse: ACCATTACCTTGTCACTGTCCATGA	188
MRP3 (ABCC3)	NM_003786	Forward: GGACTTCCAGTGCTCAGAGG Reverse: AGCTGTGGCCTCGTCTAAAA	136
MRP4 (ABCC4)	NM_005845	Forward: ATTATTGATGAAGCGACGGC Reverse: GCAAAACATACGGCTCATCA	136
MRP5 (ABCC5)	NM_001023587	Forward: CCTTTTCACTCCCTCCATCA Reverse: ACAGGTCTTGGAGCTGGAGA	136
MRP6 (ABCC6)	NM_001171	Forward: ATGAGCTTCGCAGTGTTCCT Reverse: TAGGTAGGTGGACAGGTGGC	136
MRP7 (ABCC10)	NM_001198934	Forward: GCTGGTGGGCTTGTCGCTGT Reverse: CTGGGGTTCCTGGGGCAGGT	190
OAT1 (SLC22A6)	NM_153277	Forward: CCTTGATTGGCTATGTCTACAGC Reverse: TCCATACTCAATTTGGCTCCTTC	190
OAT2 (SLC22A7)	NM_006672	Forward: CCCTTCCAACTGCGGAATGT Reverse: CTGGGGATAGGCAAAGCGG	190
OAT3 (SLC22A8)	NM_004254	Forward: AGCACCGTCATCTTGAATGTG Reverse: AGGTGTAGCAGTACCCGAGTG	190
OCT1 (SLC22A1)	NM_003057	Forward: ACGGTGGCGATCATGTACC Reverse: CCCATTCTTTTGAGCGATGTGG	190
OCT2 (SLC22A2)	NM_003058	Forward: AGACAGTGTAGGCGCTACGA Reverse: GTTAAACTCGGTGACGATGGAC	190
OCT3 (SLC22A3)	NM_021977	Forward: ATCGTCAGCGAGTTTGACCTT Reverse: ACCTGTCTGCTGCATAGCCTA	190
CNT1 (SLC28A1)	NM_004213	Forward: CCTCACCTGTGTGGTCCTCA Reverse: AGACCCCTCTTAAACCAGAGC	190
ENT1 (SLC29A1)	NM_001078176	Forward: TGAGCGGAACTCTCTCAGTG Reverse: TGAGGTAGGTGAATAACAGCAGG	190
ENT2 (SLC29A2)	NM_001532	Forward: TCAGTGCAGTCCTACAGGG Reverse: GGCGTGATAAAGTACCCCAGG	190
ENT3 (SLC29A3)	NM_001174098	Forward: TTGAGAGCTACCTTGCCGTTG Reverse: CAGTGCAGTTATCACCATGAAGA	190
OATP-B (SLCO2B1)	NM_001145211	Forward: TTTGCCCACAACAGCAACTC Reverse: TGGTTAATGTCCACATAAAGGCG	190
OATP-D (SLCO3A1)	NM_001145044	Forward: GTTCCTGACCCACCAGTACAA Reverse: CACAGGGGTAGCACCGATG	190
OATP-E (SLCO4A1)	NM_016354	Forward: CTGCTCGCCCGTCTACATTG Reverse: CCGAGGGTAACCAAGGATGG	190
Human β_2-microglobulin	NM_004048	Forward: CCAGCAGAGAATGGAAAGTC Reverse: CATGTCTCGATCCCACTTAAC	188

P-gp, P-glycoprotein; BCRP, breast cancer resistance protein; MRP, multidrug resistance associated protein; OAT, organic anion transporters; OCT, organic cation transporter; CNT, concentrative nucleoside transporters; ENT, equilibrative nucleoside transporter; OATP, organic anion-transporting polypeptide.

2.2.6 Real-time RT-PCR

Real-time RT-PCR was performed in a CFX Touch 96 instrument (Biorad) using Ssofast Evergreen mastermix (Biorad). The PCR program consisted of an initial denaturation of 95°C × 30 s, and 40 cycles of 95°C × 5 s and 60°C × 5 s, followed by a melt curve analysis heating from 65°C to 95°C with 0.5 °C increments. The primer information for transporters and internal control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) of human, macaque, rabbit, and mouse are listed in Tables 2.2 to 2.4. For many primer pairs obtained from online databases (Tables 2.2 to 2.4), the product size has been confirmed by other researchers. Some of the primers used in real-time PCR of human transporters were previously used in conventional PCR, including the primer pairs for ABCB1 (P-gp), ABCC5 (MRP5), ABCC10 (MRP7), SLC22A6 (OAT1), SLC22A8 (OAT3), SLC22A2 (OCT2) and SLC29A1 (ENT1). The size of the amplification products using these primers has also been confirmed in the conventional RT-PCR electrophoresis. The primer sequences for nuclear receptors (NRs) of human and mouse are listed in Tables 2.5 and 2.6. The primers for human genes were also used to detect macaque transporters, NRs, and GAPDH, due to the high sequence similarity between human and macaque in these genes. The efficiency of PCR reactions was confirmed using the relative standard curve method with serially diluted liver or colon cDNA. The mRNA levels of transporters were normalized to that of GAPDH using the $2^{-\Delta Ct}$ method, for the comparison of transporter levels across different tissues.

Table 2.2 Primer information for real-time RT-PCR of transporters and GAPDH in human and macaque tissues.

Common Gene name (Official gene symbol)	GeneBank accession no.	Primer sequence 5' to 3'	Ref.
P-gp (ABCB1)	NM_000927	Forward: CCCATCATTGCAATAGCAGG Reverse: TGTTCAAACTTCTGCTCCTGA	188

BCRP (ABCG2)	NM_004827	Forward: CAGGTCTGTTGGTCAATCTCACA Reverse: TCCATATCGTGGAATGCTGAAG	122
MRP4 (ABCC4)	NM_005845	Forward: AAGTGAACAACCTCCAGTTCCAG Reverse: GGCTCTCCAGAGCACCATCT	136
MRP5 (ABCC5)	NM_001023587	Forward: CCTTTTCACTCCCTCCATCA Reverse: ACAGGTCTTGGAGCTGGAGA	136
MRP7 (ABCC10)	NM_001198934	Forward: GCTGGTGGGCTTGTCGCTGT Reverse: CTGGGGTTCCTGGGGCAGGT	190
OAT1 (SLC22A6)	NM_153277	Forward: CCTTGATTGGCTATGTCTACAGC Reverse: TCCATACTCAATTTGGCTCCTTC	190
OAT3 (SLC22A8)	NM_004254	Forward: AGCACCGTCATCTTGAATGTG Reverse: AGGTGTAGCAGTACCCGAGTG	190
OCT2 (SLC22A2)	NM_003058	Forward: AGACAGTGTAGGCGCTACGA Reverse: GTTAAACTCGGTGACGATGGAC	190
ENT1 (SLC29A1)	NM_001078176	Forward: TGAGCGGAACTCTCTCAGTG Reverse: TGAGGTAGGTGAATAACAGCAGG	190
GAPDH	NM_001256799	Forward: GGAGCGAGATCCCTCCAAAAT Reverse: GGCTGTTGTCATACTTCTCATGG	190

Ref., references.

Table 2.3 Primer information for real-time RT-PCR of transporters and Gapdh in rabbit tissues.

Common Gene name (Official gene symbol)	GeneBank accession no.	Primer sequence 5' to 3'	Ref.
P-gp (Abcb1)	AY360144	Forward: CCGGAACAGTGCTATTGGAT Reverse: GATGCTGCAGTCAAACAGGA	191
BCRP (Abcg2)	XM_002716965	Forward: GCTCATTACGGATCCTTCCA Reverse: GGAATACCGAGGCTGATGAA	191
MRP4 (Abcc4)	XM_002713003	Forward: GATCTTCCTGGCAAAATGGA Reverse: ACCAATTGTCTCTGCCCAAC	191
MRP5 (Abcc5)	XM_002716488	Forward: TGTCTACATCCAGGCTGCTG Reverse: TCGAGTCACTGTGCTGTTCC	191
MRP7 (Abcc10)	XM_002714691	Forward: CTGAGCCATGGTCTGCTGTA Reverse: GGGTCACCTTCCGTAACTCA	191
OAT1 (Slc22a6)	AJ242871	Forward: AAGCTTGTGTGCTTCCTCGT Reverse: GACAACCCTTTCCCAGAACA	191
OAT3 (Slc22a8)	AF533644	Forward: TCTCCCCACTGGTGAAAATC Reverse: GGCGACAGTCCCGTAGATAA	191
Gapdh	NM_001082253	Forward: ATCACTGCCACCCAGAAGAC Reverse: GTGAGTTTCCCGTTCAGCTC	191

Ref., references.

Table 2.4 Primer information for real-time RT-PCR of transporters and Gapdh in mouse tissues.

Common Gene name (Official gene symbol)	GeneBank accession no.	Primer sequence 5' to 3'	Ref.
P-gp (Abcb1a)	NM_011076	Forward: CCATCAGCCCTGTTCTTGGAC Reverse: TCCCCAGCCTTTTAGCTTCTT	190
P-gp (Abcb1b)	NM_011075	Forward: CTGTTGGCGTATTTGGGATGT Reverse: CAGCATCAAGAGGGGAAGTAATG	190
Bcrp (Abcg2)	NM_011920	Forward: AAATGCTGTTCAGGTTATGTGGT Reverse: TCCGACCTTAGAATCTGCTACTT	190
Mrp4 (Abcc4)	NM_001033336	Forward: GTGCGACAGAGCGCCGAAGT Reverse: CTGGGGGTGGGCGCTTCTTG	190
Mrp5 (Abcc5)	NM_176839	Forward: AGGGGGAGCTGTTAATGGAG Reverse: GTCAGAACTTACTGGTCCACTG	190
Mrp7 (Abcc10)	NM_170680	Forward: ATTGCCAGCGAGGTACATTTC Reverse: GTGCCAACCAGGCATAGGAA	190
Oat1 (Slc22a6)	NM_008766	Forward: CTGATGGCTTCCCACAACAC Reverse: GTCCTTGCTTGTCCAGGGG	190
Oat3 (Slc22a8)	NM_031194	Forward: ATGACCTTCTCCGAGATTCTGG Reverse: GTGGTTGGCTATTCCGAGGAT	190
Gapdh	NM_008084	Forward: GCCCAGAACATCATCCCTGC Reverse: CCGTTCAGCTCTGGGATGACC	192

Ref., references.

Table 2.5 Information of the primers used for the real-time RT-PCR of nuclear receptors (NRs) in human and macaque tissues.

Common Gene name	GeneBank accession number	Primer sequences 5' to 3'	Ref.
PXR	NM_022002	Forward: GGCCACTGGCTATCACTTCAA Reverse: TTCATGGCCCTCCTGAAAA	193
CAR	NM_001077474	Forward: GATGCTGGCATGAGGAAAGAC Reverse: TTGCTCCTTACTCAGTTGCAC	190
VDR	NM_001017536	Forward: TCTCCAATCTGGATCTGAGTGAA Reverse: GGATGCTGTAACTGACCAGGT	190
PPAR-α	NM_005036	Forward: ATGGTGGACACGGAAAGCC Reverse: CGATGGATTGCGAAATCTCTTGG	190
PPAR-β	NM_177435	Forward: TCACACAGTGGCTTCTGCTC Reverse: TGAACGCAGATGGACCTCTA	194
PPAR-γ	NM_138711	Forward: AAGGCCATTTTCTCAAACGA Reverse: GAGAGATCCACGGAGCTGAT	194
ER-α	NM_000125	Forward: ATGATCAACTGGGCGAAGAG Reverse: CAGGATCTCTAGCCAGGCAC	194
ER-β	NM_001214902	Forward: TCCATCGCCAGTTATCACATCT	190

		Reverse: CTGGACCAGTAACAGGGCTG	
PR	M15716	Forward: GTCAGTGGGCAGATGCTGTA Reverse: TGCCACATGGTAAGGCATAA	191
AR	NM_000044	Forward: TTGTGTCAAAAGCGAAATGG Reverse: AGTCAATGGGCAAAACATGG	194
GR	NM_001204264	Forward: ACAGCATCCCTTTCTCAACAG Reverse: AGATCCTTGGCACCTATTCCAAT	190
MR	NM_000901	Forward: GAAGTGATGGGTATCCGGTC Reverse: TTTGAAGGTCTTGAAGATCCAG	194
RAR-α	NM_000964	Forward: AAGCCCGAGTGCTCTGAGA Reverse: TTCGTAGTGTATTTGCCCAGC	190
RAR-β	NM_000965	Forward: TCCAGAAGTGCTTTGAAGTGG Reverse: CTCTGTGCATTCTTGCTTCG	194
RAR-γ	NM_000966	Forward: TTTCGAGATGCTGAGCCCTA Reverse: TCTGAGCTGGTGCTCTGTGT	194
RXR-α	NM_002957	Forward: GACGGAGCTTGTGTCCAAGAT Reverse: AGTCAGGGTTAAAGAGGACGAT	190
RXR-β	NM_021976	Forward: ACGGCTATGTGCAATCTGC Reverse: CGGATGGTGCGTTTGAAGAA	190
RXR-γ	NM_006917	Forward: GGAGAGGAACATGAACTGACG Reverse: ATGGATGTAGAGCCAGTGTGG	194
ROR-α	NM_134261	Forward: ACTCCTGTCCTCGTCAGAAGA Reverse: CATCCCTACGGCAAGGCATTT	190
ROR-β	NM_006914	Forward: CTGGGAGCAGCTTCATGACT Reverse: ATCCCAGAGGACTTATCGCC	194
ROR-γ	NM_001001523	Forward: CTGCTGAGAAGGACAGGGAG Reverse: CGACTTGTCCCCACAGATTT	194
Nrf2	NM_006164	Forward: ACACGGTCCACAGCTCATC Reverse: TCTTGCCTCCAAAGTATGTCAA	194
AhR	NM_001621	Forward: TCAGTTCTTAGGCTCAGCGTC Reverse: AGTTATCCTGGCCTCCGTTT	194

PXR: pregnane X receptor*;* CAR: Constitutive androstane receptor; VDR: Vitamin D receptor; PPAR: Peroxisome proliferator-activated receptor; ER: Estrogen receptor; PR: Progesterone receptor; AR: Androgen receptor; GR: Glucocorticoid receptor; MR: Mineralocorticoid receptor; RAR: Retinoic acid receptor; RXR: Retinoid X receptor; ROR: RAR-related orphan receptor; Nrf2: Nuclear factor (erythroid-derived 2)-like 2; AhR: Aryl hydrocarbon receptor. Ref., references.

Table 2.6 Information of the primers used for the real-time RT-PCR of nuclear receptors (NRs) in mouse tissues.

Common Gene name	GeneBank accession number	Primer sequences 5' to 3'	Ref.
Mouse PXR	NM_010936	Forward: GATGGAGGTCTTCAAATCTGCC	190

		Reverse: CAGCCGGACATTGCGTTTC	
Mouse CAR	NM_001243062	Forward: TTCAAGCCTCCGGCCTATCT Reverse: TGATCTGTTGCACCATAAACGTG	190
Mouse VDR	NM_009504	Forward: ACCCTGGTGACTTTGACCG Reverse: GGCAATCTCCATTGAAGGGG	190
Mouse PPAR-α	NM_011144	Forward: AGAGCCCCATCTGTCCTCTC Reverse: ACTGGTAGTCTGCAAAACCAAA	190
Mouse PPAR-β	NM_011145	Forward: TCCATCGTCAACAAAGACGGG Reverse: ACTTGGGCTCAATGATGTCAC	190
Mouse PPAR-γ	NM_001127330	Forward: GGAAGACCACTCGCATTCCTT Reverse: GTAATCAGCAACCATTGGGTCA	190
Mouse ER-α	NM_007956	Forward: CCTCCCGCCTTCTACAGGT Reverse: CACACGGCACAGTAGCGAG	190
Mouse ER-β	NM_010157	Forward: CTGTGATGAACTACAGTGTTCCC Reverse: CACATTTGGGCTTGCAGTCTG	190
Mouse PR	NM_008829	Forward: CTCCGGGACCGAACAGAGT Reverse: ACAACAACCCTTTGGTAGCAG	190
Mouse AR	NM_013476	Forward: CTGGGAAGGGTCTACCCAC Reverse: GGTGCTATGTTAGCGGCCTC	190
Mouse GR	NM_008173	Forward: AGCTCCCCCTGGTAGAGAC Reverse: GGTGAAGACGCAGAAACCTTG	190
Mouse MR	NM_001083906	Forward: GAAAGGCGCTGGAGTCAAGT Reverse: TGTTCGGAGTAGCACCGGAA	190
Mouse RAR-α	NM_009024	Forward: TTCTTTCCCCCTATGCTGGGT Reverse: GGGAGGGCTGGGTACTATCTC	190
Mouse RAR-β	NM_011243	Forward: CTGCTCAATCCATCGAGACAC Reverse: CTTGTCCTGGCAAACGAAGC	190
Mouse RAR-γ	NM_001042727	Forward: ATGTACGACTGCATGGAATCG Reverse: CCAGTGGCTCTGCGTAGTAA	190
Mouse RXR-α	NM_011305	Forward: ATGGACACCAAACATTTCCTGC Reverse: CCAGTGGAGAGCCGATTCC	190
Mouse RXR-β	NM_011306	Forward: CCACCTCTTACCCCTTCAGC Reverse: TGGAAGAACTGATGACTGGGA	190
Mouse RXR-γ	NM_009107	Forward: CATGAGCCCTTCAGTAGCCTT Reverse: CGGAGAGCCAAGAGCATTGAG	190
Mouse ROR-α	NM_013646	Forward: GTGGAGACAAATCGTCAGGAAT Reverse: TGGTCCGATCAATCAAACAGTTC	190
Mouse ROR-β	NM_146095	Forward: GCAGCATTAGCAATGGCCTC Reverse: GACGGCTGACCGGAATCTATG	190
Mouse ROR-γ	NM_011281	Forward: GACCCACACCTCACAAATTGA Reverse: AGTAGGCCACATTACACTGCT	190
Mouse Nrf2	NM_010902	Forward: TAGATGACCATGAGTCGCTTGC Reverse: GCCAAACTTGCTCCATGTCC	190
Mouse AhR	NM_013464	Forward: AGCCGGTGCAGAAAACAGTAA Reverse: AGGCGGTCTAACTCTGTGTTC	190

2.2.7 Statistical methods

Statistical analyses were conducted using the GraphPad Prism software. One way analysis of variance (ANOVA) with Bonferroni post-hoc test was used to compare the mRNA level of a given transporter across different tissue types in a species. $P < 0.05$ was considered as statistically significant, and $P < 0.01$ was considered as very significant.

2.3 Results

2.3.1 Conventional RT-PCR screening of efflux and uptake transporters in human cervicovaginal tissues

Since the transporter expression profile remains largely unknown for human cervicovaginal tissues, conventional RT-PCR was used to quickly screen the transporters most relevant to antiretroviral drug pharmacokinetics. Nine of the efflux transporters from the ABC superfamily were examined and the results were summarized in Table 2.7. The liver samples had highest expression of MRP2, MRP3 and MRP6, followed by P-gp, BCRP, MRP1, MRP5, MRP5 and MRP7 with moderate or low expression level. Compared to the liver, P-gp, BCRP, MRP1, MRP4, MRP5 and MRP7 were moderately or highly expressed efflux transporters in human cervicovaginal tissues. Inter-individual difference in the expression level of P-gp, BCRP, MRP1, MRP4 and MRP7 was observed. The expression level of P-gp, BCRP, and MRP4 appeared to be different between epithelium and stroma. Ectocervical MRP1 and MRP4 level appeared to be higher than those in vagina (Figure 2.1). Thirteen uptake transporters were examined, including 10 from the SLC superfamily and 3 from the SLCO superfamily.

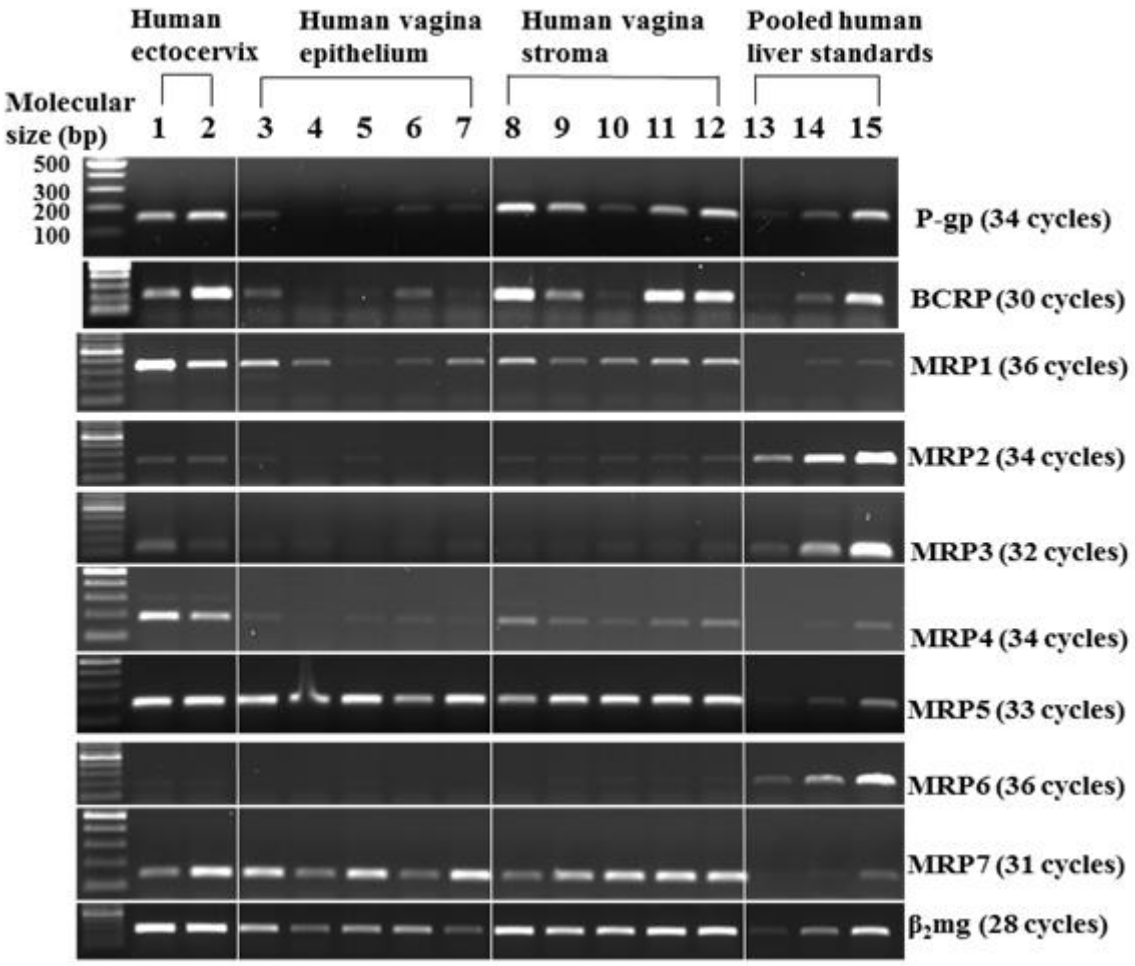


Figure 2.1 Conventional RT-PCR screening of efflux transporters in human cervicovaginal tissues.

Lanes 1 and 2: ectocervical epithelium and stroma pooled from 7 patients. Lanes 3-7: vaginal epithelium from 5 individual patients. Lanes 8-12: vaginal stroma from the same 5 patients. Fifty ng cDNA for each sample was loaded for PCR. Lanes 13-15: serially diluted standards including human liver cDNA transcribed from 1, 5, 25 ng of liver total RNA, which was extracted from liver samples pooled from 6 donors. PCR cycle numbers are indicated in the parentheses. β2-microglobulin (β2mg) was used as internal control to indicate cDNA loading in PCR.

Table 2.7 Summary of efflux transporters expression in human cervicovaginal tissues.

Transporter	Human transporter level (% of human liver)	
	Human ectocervix	Human vagina
P-gp (ABCB1)	++	++
BCRP (ABCG2)	+++	+++
MRP1 (ABCC1)	++++	+++
MRP2 (ABCC2)	-	-
MRP3 (ABCC3)	-	-
MRP4 (ABCC4)	++++	+++
MRP5 (ABCC5)	++++	++++
MRP6 (ABCC6)	-	-
MRP7 (ABCC10)	++++	++++

The density of PCR bands generated from ectocervix and vagina cDNA were compared to the bands generated from liver cDNA, and the expression levels of efflux transporters relative to liver levels were summarized in the table above. -, ≤2% or undetectable; +, means 2-10%, ++, 10-50%; +++, 50-100%; ++++, >>100%. The corresponding gel images were shown in Figure 2.1.

Among all the tested uptake transporters, SLC22A7 (OAT2), SLC22A2 (OCT2), SLC22A3 (OCT3), SLC29A1 (ENT1), SLCO3A1 (OATP-D) and SLCO4A1 (OATP-E) were the most highly expressed compared to their expression in liver. The discrepancy in the expression level was observed for SLC22A7 (OAT2), SLC22A2 (OCT2), SLC22A3 (OCT3), SLC29A1 (ENT1) and SLCO3A1 (OATP-D) between epithelium and stroma in ectocervix. The discrepant expression was also observed for OCT3 and OATP-E between epithelium and stroma in vagina (Figure 2.2). Results were summarized in Table 2.8.

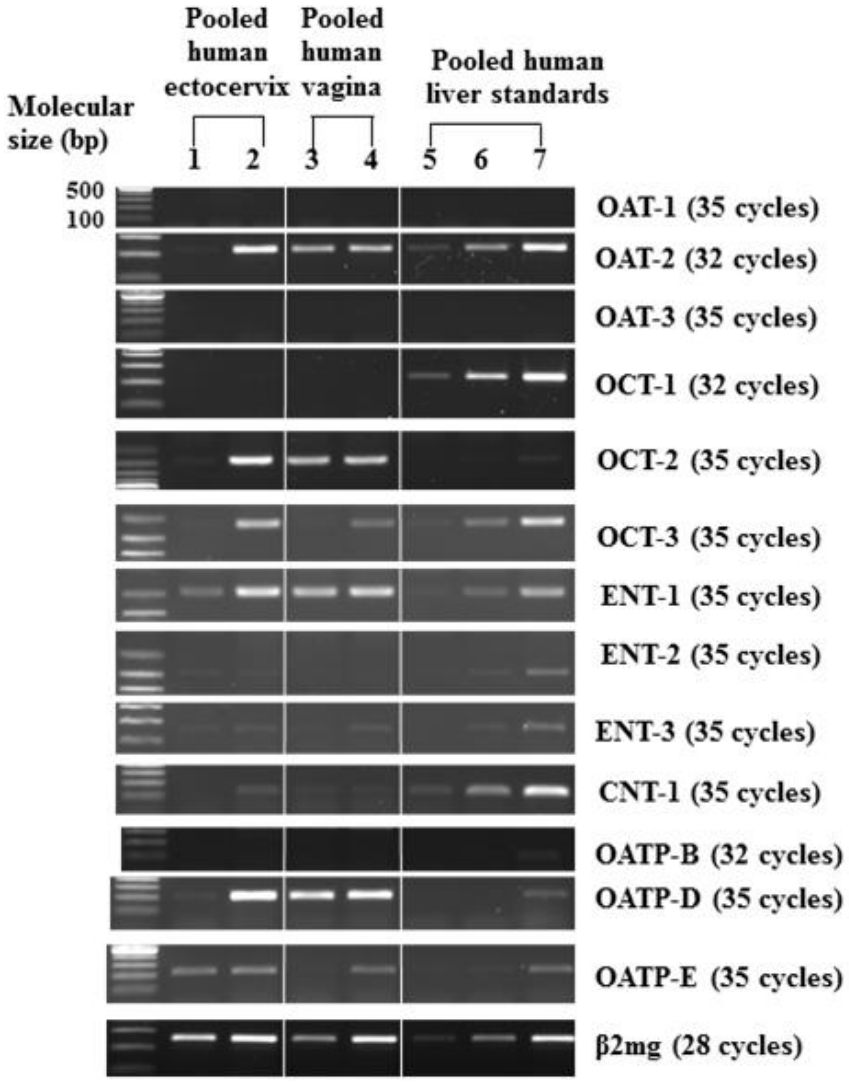


Figure 2.2 Conventional RT-PCR screening of uptake transporters in human cervicovaginal tissues

Lanes 1 and 2: ectocervical epithelium and stroma pooled from 7 patients. Lanes 3 and 4: vaginal epithelium and stroma pooled from 5 patients. Lanes 5-7: serially diluted pooled human liver cDNA transcribed from 1, 5, 25 ng of total RNA. The cDNA loading is 50 ng for cervicovaginal tissue samples (Lanes 1-4), and 1, 5, 25 ng for liver standards (Lanes 5-7). PCR cycle numbers are indicated in the parentheses.

Table 2.8 Summary of uptake transporters expression in human cervicovaginal tissues.

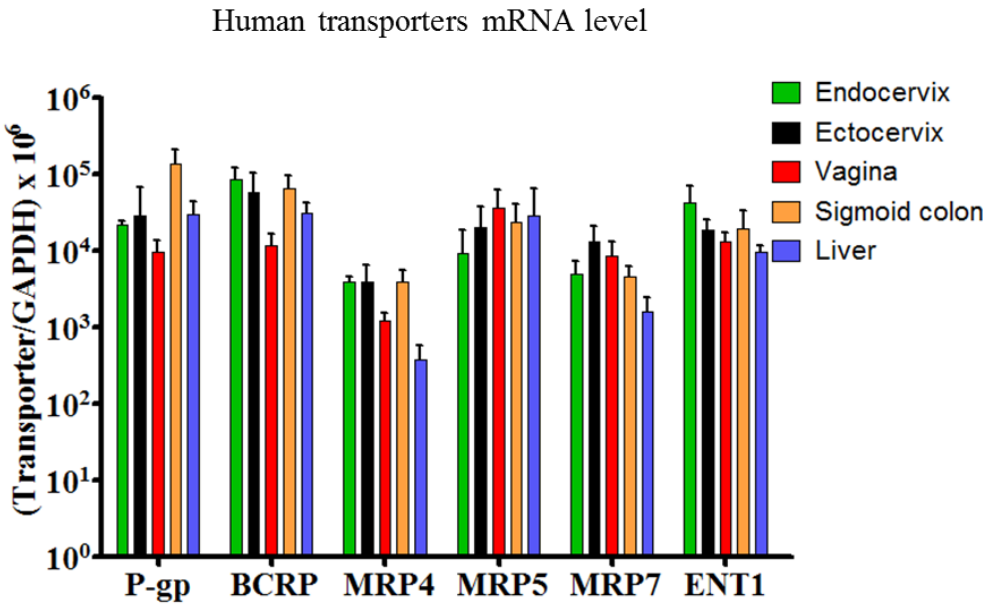
Transporter	Human transporter level (% of human liver)	
	Human ectocervix	Human vagina
OAT1 (SLC22A6)	-	-
OAT2 (SLC22A7)	++	++
OAT3 (SLC22A8)	-	-
OCT1 (SLC22A1)	-	-
OCT2 (SLC22A2)	++++	++++
OCT3 (SLC22A3)	++	+
ENT1 (SLC29A1)	+++	+++
ENT2 (SLC29A2)	-	-
ENT3 (SLC29A3)	-	-
CNT1 (SLC28A1)	-	-
OATP-B (SLCO2B1)	-	-
OATP-D (SLCO3A1)	++++	++++
OATP-E (SLCO4A1)	++	++

The density of PCR bands generated from ectocervix and vagina cDNA were compared to the bands generated from liver cDNA, and the expression levels of efflux transporters relative to liver levels were summarized in the table above. -, $\leq$2% or undetectable; +, means 2-10%, ++, 10-50%; +++, 50-100%; ++++, >>100%. The corresponding gel images were shown in Figure 2.2.

2.3.2 Real-time RT-PCR examination of mRNA levels of a select panel of transporters in human cervicovaginal and colorectal tissues

To confirm the conventional RT-PCR results, and quantitatively compare transporter expression across different tissues, real-time PCR was conducted to examine mRNA levels of the most highly expressed and most relevant transporters identified in conventional PCR (P-gp, BCRP, MRP4, 5, 7, OAT1, 3, OCT2, ENT1). The general tendency of transporter expression found in human lower genital tract and colorectal tissue was that P-gp, BCRP, MRP4, 5, 7 and ENT1

were moderately to highly expressed compared to GAPDH in cervicovaginal and colorectal tissues (between 1/1000 and 1/10 of GAPDH level), and compared to the transporter levels in the positive control tissue human liver (Figure 2.3). On the contrary, OAT1, OAT3 and OCT2 were not detectable (data not shown). These results were generally in line with the conventional PCR results except OCT2.



Tissue mRNA levels relative to human liver level				
Human transporters	Endocervix	Ectocervix	Vagina	Sigmoid colon
P-gp	0.73±0.08	0.97±1.31	0.32±0.13	4.62±2.50
BCRP	2.77±1.16	1.92±1.42	0.39±0.16	2.10±1.05
MRP4	10.45±1.75	10.31±6.73	3.13±0.87	10.35±4.31
MRP5	0.34±0.29	0.71±0.57	1.07±0.45	0.84±0.61
MRP7	2.60±1.70	7.28±5.22	4.60±2.44	2.88±0.95
ENT1	3.79±2.84	1.74±0.90	1.17±0.61	2.08±1.50

Figure 2.3 Real-time RT-PCR analysis of a select panel of transporters in human cervicovaginal and colorectal tissues

The expression in endocervix, ectocervix, vagina, sigmoid colon and liver were examined in tissues from 4-6 human donors (Endocervix, 4 donors; ectocervix, 6 donors; vagina, 5 donors; sigmoid colon, 5 donors; liver, 6 donors). Only one tissue sample was obtained from each donor, and a small piece (50-200 mg) cut from each tissue sample was used for RNA extraction and RT-PCR analysis. The threshold cycle numbers (Ct) of transporters and GAPDH of each sample

were measured in triplicate, and the average Ct was used to reflect the Ct of a tested transporter or GAPDH in this sample. The GAPDH-normalized transporter levels (generated using $2^{-\Delta Ct}$ method) in different tissues were plotted in the figure shown above. the GAPDH-normalized transporter expression levels in genital tract and colorectal tissues relative to the GAPDH-normalized transporter expression levels in human liver were summarized in the table. The data shown represent mean ± standard deviation of all samples in a given group.

Statistical analyses were conducted to compare the expression of each transporter across different types of human tissues. Each of the genital tract tissues (endocervix, ectocervix, vagina) displayed several fold lower (statistically significant) expression level in P-gp compared to the sigmoid colon, but there was no difference between genital tract tissues and liver in P-gp expression (Figure 2.3). Sigmoid colon displayed several fold higher P-gp level compared to liver ($p < 0.01$). Within human lower genital tract, P-gp expression was highest in ectocervix, followed by endocervix and vagina, but there was no significant difference among these three tissue types (Figure 2.3).

For BCRP, there was no difference between genital tract tissues and colorectal/liver tissues. The colorectal BCRP level was significantly higher than the liver level ($p < 0.05$). Within the genital tract, human BCRP expression was highest in endocervix, followed by ectocervix and vagina, and there was a significant difference between endocervix and vagina ($p < 0.01$) (Figure 2.3).

For MRP4, the mRNA levels in human endocervix and ectocervix were found to be significantly higher than human liver level ($p < 0.001$ and $p < 0.01$, respectively). However, no difference existed between genital tract and colon. Within the genital tract, the human MRP4 expression in endocervix and ectocervix were similar, while vaginal MRP4 level was significantly lower than

the endocervical level ($p < 0.001$). The colorectal MRP4 level was significantly higher than the liver level ($p < 0.001$).

For MRP5, no statistical difference was found between genital tract tissues, colorectal tissue, and liver. For MRP7, the ectocervical level was significantly higher than the colon ($p = 0.05$) and liver ($p < 0.01$), but there was no difference between other pairs of tissue types. For ENT1, the endocervical level was significantly higher than the liver level ($p < 0.05$) and vaginal level ($p = 0.05$), while there was no significant difference between any other pair of tissue types.

For each transporter, the GAPDH-normalized mRNA levels in human cervicovaginal and colorectal tissues were divided by the average of GAPDH-normalized transporter level in human liver, and the results were shown in the table embedded in Figure 2.3. It should be noticed that healthy human liver has low level of MRP4 gene expression, although the expression can be profoundly up-regulated by the treatment of toxicants.[195,196] Therefore, cervicovaginal and colorectal tissue MRP4 was expressed at moderate level compared to GAPDH (>1/1000 of GAPDH), but when normalizing these levels to the MRP4 level in healthy liver, high ratios could be obtained. The function of MRP4 in cervicovaginal and colorectal tissues cannot be assumed from these high ratios, and should be experimentally confirmed.

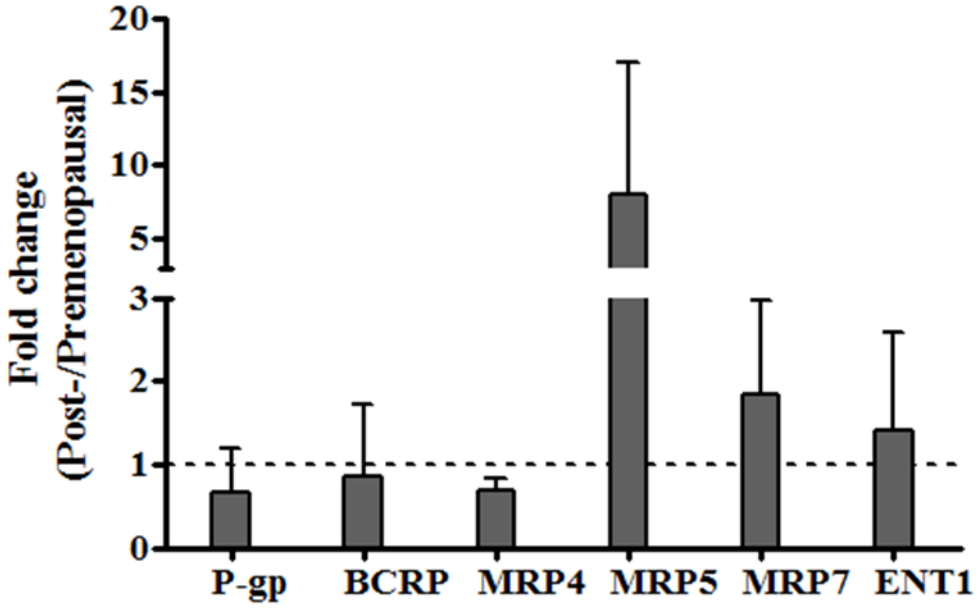
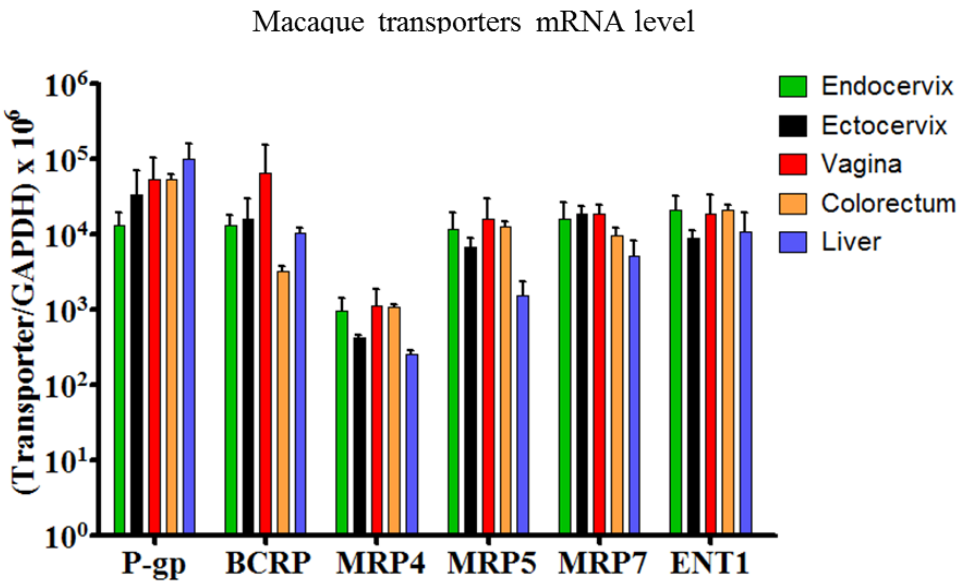


Figure 2.4 Effect of menopause on the mRNA expression of transporters in human ectocervix.
GAPDH-normalized transporter expression levels were compared between premenopausal and postmenopausal human ectocervix tissues. Tissues were collected from 12 patients (6 premenopausal and 6 postmenopausal women). For each transporter tested, the GAPDH-normalized transporter levels from the 6 donors in the postmenopausal group were compared with the average of GAPDH-normalized transporter levels in the premenopausal group. The postmenopausal/premenopausal ratios (mean ± standard deviation) were plotted above.

The effect of menopause on transporter mRNA expression was examined by comparing transporter levels in pre- and postmenopausal human ectocervix (Figure 2.4). Although MRP5 level was approximately 8-fold higher in postmenopausal tissues, no significant difference was found for MRP5 or another transporter examined, due to large inter-individual variability in MRP5 mRNA levels.

2.3.3 Real-time RT-PCR examination of mRNA levels of a select panel of transporters in pigtailed macaque cervicovaginal and colorectal tissues

The same panel of transporters was examined in pigtailed macaque tissues. In macaques, the six transporters were also moderately to highly expressed in endocervix, ectocervix, vagina, and colorectum compared to macaque liver levels (Figure 2.5). OAT1 and OAT3 were also examined in macaque tissues but were not detectable by real-time RT-PCR. For each of the transporters examined in macaque, there was no significant difference in transporter mRNA level among endocervix, ectocervix and vagina, colorectum, nor was there any significant difference between these tissues and positive control tissue (liver), although the MRP4, MRP5 and MRP7 levels in these tissues were several times higher than the corresponding liver levels (Figure 2.5). For each transporter, the GAPDH-normalized mRNA levels in macaque cervicovaginal and colorectal tissues were divided by the average of GAPDH-normalized transporter level in macaque liver, and the results were shown in the table embedded in Figure 2.5.



Tissue mRNA levels relative to macaque liver level				
Macaque transporters	Endocervix	Ectocervix	Vagina	Colorectum
P-gp	0.13±0.06	0.33±0.36	0.53±0.52	0.54±0.09
BCRP	1.28±0.42	1.55±1.25	6.14±8.44	0.31±0.05
MRP4	3.78±1.63	1.61±0.18	4.26±3.05	4.24±0.36
MRP5	7.39±4.94	4.28±1.49	10.18±8.95	8.04±1.53
MRP7	3.04±2.03	3.53±0.94	3.59±1.19	1.82±0.47
ENT1	1.97±0.96	0.83±0.21	1.69±1.46	1.90±0.34

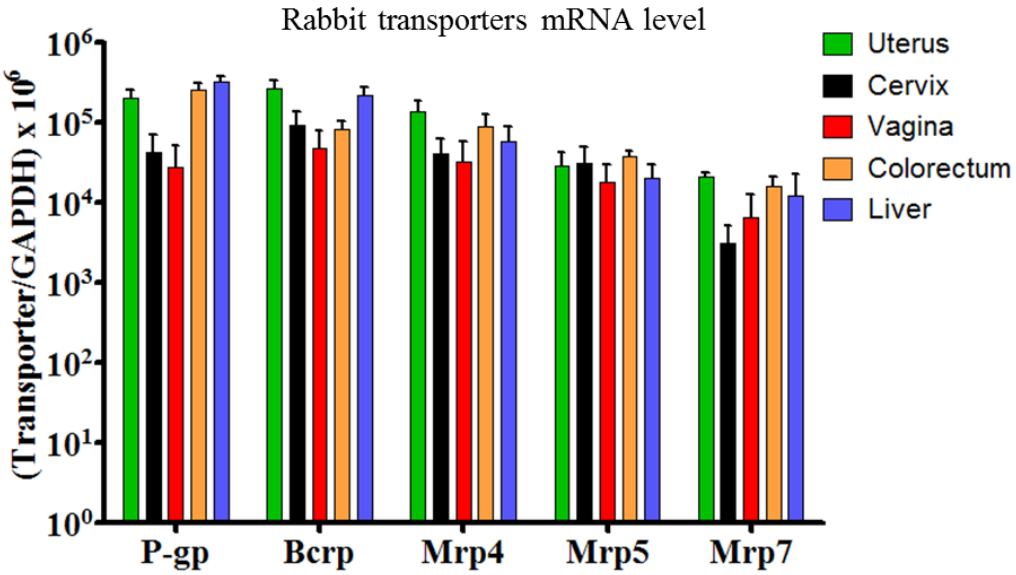
Figure 2.5 Real-time RT-PCR analysis of a select panel of transporters in macaque cervicovaginal and colorectal tissues

The expression in endocervix, ectocervix, vagina, colorectum and liver were examined in tissues from 3 macaques. For each macaque, all the 5 kinds of tissues were collected. A small piece (50-200 mg) cut from each tissue sample was used for RNA extraction and RT-PCR analysis. The threshold cycle numbers (Ct) of transporters and Gapdh of each sample were measured in triplicate, and the average Ct was used to reflect the Ct of transporter or Gapdh in this sample. The Gapdh-normalized transporter levels (generated using $2^{-\Delta Ct}$ method) in different tissues were plotted in the figure shown above. The Gapdh-normalized transporter expression levels in genital tract and colorectal tissues relative to the Gapdh-normalized transporter expression levels in macaque liver were summarized in the table. The data shown represent mean ± standard deviation of all samples in a given group.

Overall, P-gp, BCRP, MRP4,5,7 and ENT1 were moderately to highly expressed at the mRNA level in the lower genital tract and colorectal tissues of humans and macaques, compared to

macaque Gapdh in corresponding tissues (between 1/1000 and 1/10 of GAPDH level), and compared to the transporter levels in macaque liver. Oat1, Oat3, and Oct2 were not detectable in all kinds of macaque tissues tested (data not shown). The cervicovaginal and colorectal expression patterns of these transporters were generally comparable between human and macaque (Figures 2.3 and 2.5).

2.3.4 Real-time RT-PCR examination of mRNA levels of a select panel of transporters in rabbit cervicovaginal and colorectal tissues



Tissue mRNA levels relative to rabbit liver level				
Rabbit transporters	Uterus	Cervix	Vagina	Colorectum
P-gp	0.63±0.18	0.13±0.09	0.09±0.08	0.80±0.18
Bcrp	1.23±0.37	0.43±0.22	0.22±0.15	0.39±0.10
Mrp4	2.36±0.91	0.72±0.40	0.57±0.44	1.59±0.65
Mrp5	1.42±0.70	1.57±0.94	0.90±0.57	1.86±0.31
Mrp7	1.72±0.16	0.27±0.16	0.35±0.41	0.92±0.67

Figure 2.6 Real-time RT-PCR analysis of a select panel of transporters in rabbit cervicovaginal and colorectal tissues.
The expression in uterus, cervix, vagina, colorectum and liver were examined in tissues from 3-7

rabbits (uterus, cervix and liver were collected from 3 rabbits; colorectum was collected from 6 rabbits; vagina was collected from 7 rabbits). For each rabbit, all the 5 kinds of tissues were collected. For vagina, colorectum and liver, a small piece (50-200 mg) cut from each tissue sample was used for RNA extraction and RT-PCR analysis. For uterus and cervix, whole tissue samples were used for analysis. Rabbit endocervix and ectocervix were difficult to distinguish, therefore the rabbit genital tract part connecting vagina and uterine horns was obtained to represent the whole cervix. The uterine horn adjacent to cervix was obtained to represent the tissue similar to endocervix. The threshold cycle numbers (Ct) of transporters and Gapdh of each sample were measured in triplicate, and the average Ct was used to reflect the Ct of transporter or Gapdh in this sample. The Gapdh-normalized transporter levels (generated using $2^{-\Delta Ct}$ method) in different tissues were plotted in the figure shown above. the Gapdh-normalized transporter expression levels in genital tract and colorectal tissues relative to the Gapdh-normalized transporter expression levels in rabbit liver were summarized in the table. The data shown represent mean ± standard deviation of all samples in a given group.

The New Zealand White rabbit is widely used to evaluate the safety of vaginally or rectally administered microbicides.[197] Since the safety/toxicity of drug products depends on drug pharmacokinetics in the tissues, it is necessary to examine transporter expression pattern in rabbit tissues in comparison to human. The anatomy of rabbit lower genital tract is quite different from that of human and macaque. In human and macaque, the endocervix is lined with single-layer epithelium, while the ectocervix and vaginal tissues are lined with stratified, squamous epithelial cells. However, in rabbit genital tract, the upper and lower genital tracts are lined with single-layer columnar epithelium, and the endocervix and ectocervix were not readily distinguishable. Therefore, rabbit uterus close to the lower genital tract was used in rabbit tissue characterization, to represent the most distal region that can be reached by male ejaculation in rabbit genital tract.

The expression of 5 efflux transporters (P-gp, Bcrp, Mrp4, 5, 7) most relevant to antiretroviral drugs were examined in rabbit tissues. In rabbits, the five transporters were also moderately to highly expressed in uterus, cervix, vagina, and colorectum compared to rabbit Gapdh in

corresponding tissues, and compared to rabbit liver transporter levels (Figure 2.6). OAT1 and OAT3 were also examined in these tissues but were not detectable by real-time RT-PCR.

For each transporter, the Gapdh-normalized mRNA levels in rabbit cervicovaginal and colorectal tissues were divided by the average of Gapdh-normalized transporter level in rabbit liver, and the results were shown in the table embedded in Figure 2.6. Overall, the liver-normalized transporter mRNA levels in rabbit genital tract and colorectum appear to be lower than those normalized levels in human and macaque. For P-gp, the mRNA level in rabbit uterus, cervix and vagina were significantly lower than liver level. For BCRP, the cervix, vagina and colorectum levels were significantly lower than the liver level.

2.3.5 Real-time RT-PCR examination of mRNA levels of a select panel of transporters in mouse tissues

The mRNA levels of P-gp, Bcrp, Mrp4, 5, 7, Oat1 and Oat3 were also examined in Swiss Webster mice undergoing natural estrous cycling, or treated with PMSG/Depo-Provera for estrous stage synchronization.

As shown in Figure 2.7, the stages of mouse estrous cycle could be clearly identified by observing mouse vaginal smears under a regular bright light microscope. Small nucleated epithelial cells constituted the major cell type in the smear from proestrus stage. Extensive amount of anucleated cornified epithelial cells could be readily observed in the smear collected at estrus stage. Three types of cells, leukocytes, cornified, and nucleated epithelial cells could be found in the smear collected at metestrus stage. A lot of leukocytes could be observed as the

predominant cell type in the smear collected at the diestrus stage. The vaginal smear collected from PMSG-treated mice was very similar to the smear collected at natural estrus stage. The smear collected from Depo-Provera treated mice was very similar to the smear collected at natural diestrus stage.

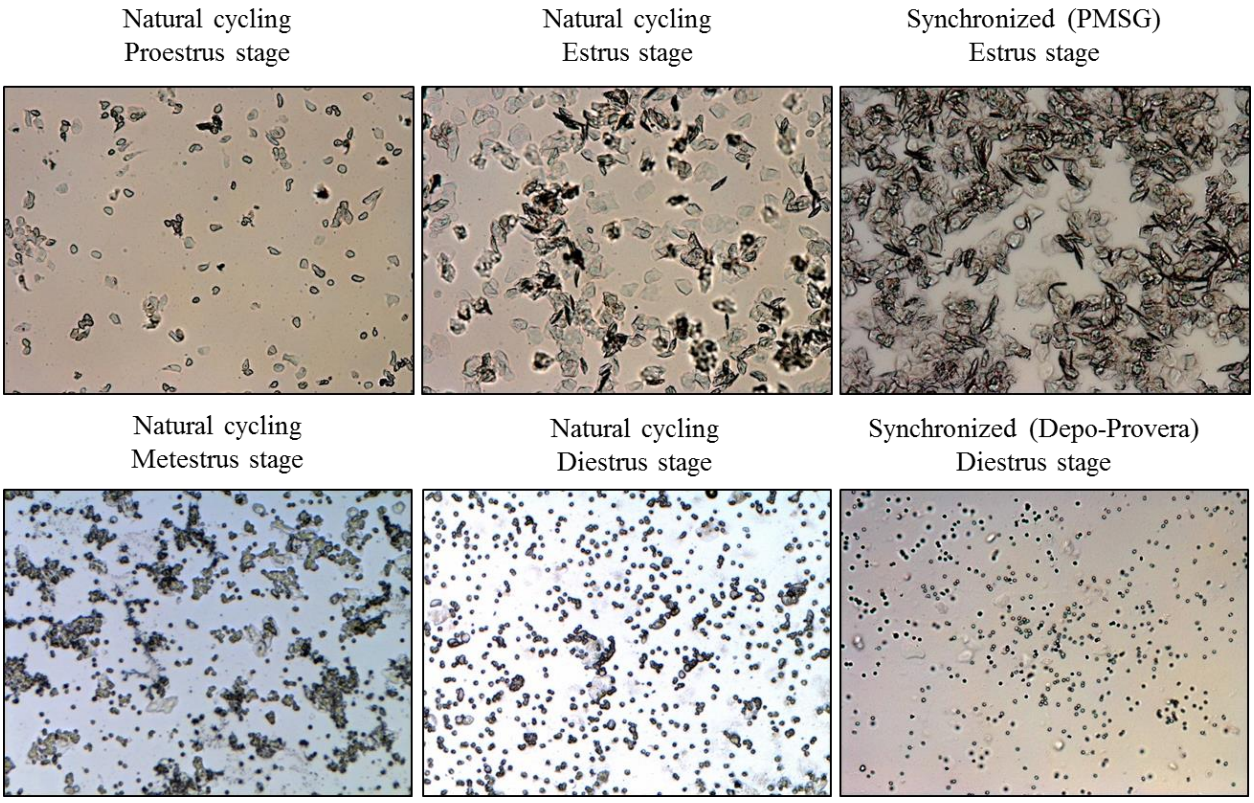


Figure 2.7 Vaginal cytology of mice undergoing natural estrous cycling or synchronized with PMSG/Depo-Provera.
At different stages of mouse estrus cycle, or after the synchronization with PMSG or Depo-Provera, vaginal smear samples were collected using 20 µL of saline. Five µL of the smear was spread on a glass slide and observed using bright light microscope. Magnification, 20 × for all pictures. The pictures shown are representative of the vaginal smear observations from more than 3 mice.

The effects of estrous cycle and exogenous treatments on cervicovaginal tissue morphology were demonstrated in Figure 2.8. During natural estrous cycle, the endocervical epithelium was a single-layer columnar cells at both estrus and diestrus stages. However, the thickness of ectocervical and vaginal epithelia underwent cyclic changes during the natural cycle. The

epithelial layers of these two tissue segments were obviously thinner at diestrus compared to estrus stage. This effect is due to the cyclic change of estrogens and progesterone during the estrous cycle. Estrogens increase the epithelial thickness while progesterone exerts the opposite effects.

After PMSG treatment, the epithelial layers of mouse endocervix, ectocervix and vagina were markedly thickened, and the cornified epithelial cells can be observed on the superficial epithelial layers. This is in line with PMSG's effect of stimulating estrogen production. On the contrary, the epithelium of mouse tissues became much thinner after Depo-Provera treatment, which was similar to that observed at natural diestrus stage (Figure 2.8).

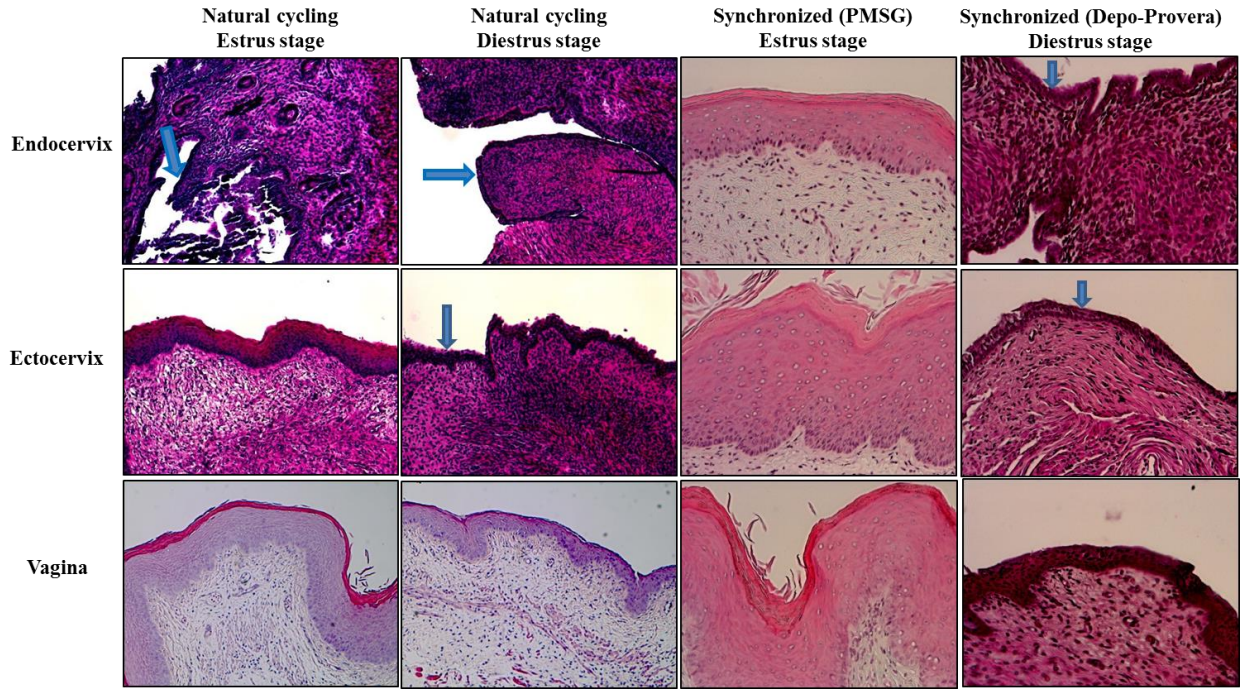


Figure 2.8 The morphology of mouse cervicovaginal tissues undergoing natural estrous cycling or synchronized with PMSG/Depo-Provera.
The tissues were collected and fixed in 10% neutral buffered formalin for no less than 24 hours, and subjected to H & E staining. For the tissue samples with thin epithelium, blue arrows were used to indicate the position of epithelium. Magnification, 20 × for all pictures. Pictures shown

were representative of tissues collected from at least 3 mice.

These vaginal cytology and tissue histology results were in line with published reports on mouse estrous cycle, and demonstrated that the synchronization protocols utilized in this study were successful in synchronizing mice into estrus and diestrus stages.

The effects of estrous cycle, PMSG and Depo-Provera on the mRNA levels of 5 efflux transporters, including Abcb1a/Abcb1b (P-gp), Abcg2 (Bcrp), Abcc4 (Mrp4), Abcc5 (Mrp5), Abcc10 (Mrp7) were demonstrated from Figures 2.9 to 2.13. The effects were tissue- and transporter-dependent, however, in mouse female genital tract, some general tendencies appeared to exist. The mRNA levels of transporters tended to be highest at diestrus stage and lowest at the estrus stage, during the natural cycle. The PMSG treatment generally decreased transporter levels, while Depo-Provera generally increased transporter mRNA levels compared to the stages during natural cycle. For example, in mouse uterus, endocervix and ectocervix, the mRNA levels of Abcc4 (Mrp4) (Figure 2.11) were higher at diestrus stage compared to estrus. PMSG and Depo-Provera synchronization significantly altered Abcc4 level compared to the natural estrous cycle. Abcc4 levels in uterus, endocervix and ectocervix were significantly decreased after PMSG synchronization, compared to the estrus and diestrus stages of the natural cycle. On the contrary, after Depo-Provera synchronization, Abcc4 levels in mouse uterus, endocervix and ectocervix were significantly higher than both stages of the natural cycle, and Abcc4 level in mouse vagina was significantly higher than the estrus stage of the natural cycle.

PMSG treatment significantly decreased Abcc4 level in uterus, endocervix and ectocervix, compared to either stage during the natural cycle. On the contrary, Depo-Provera treatment

significantly increased Mrp4 levels in all the four segments of mouse genital tract compared to the levels during natural estrous cycle (Figure 2.11).

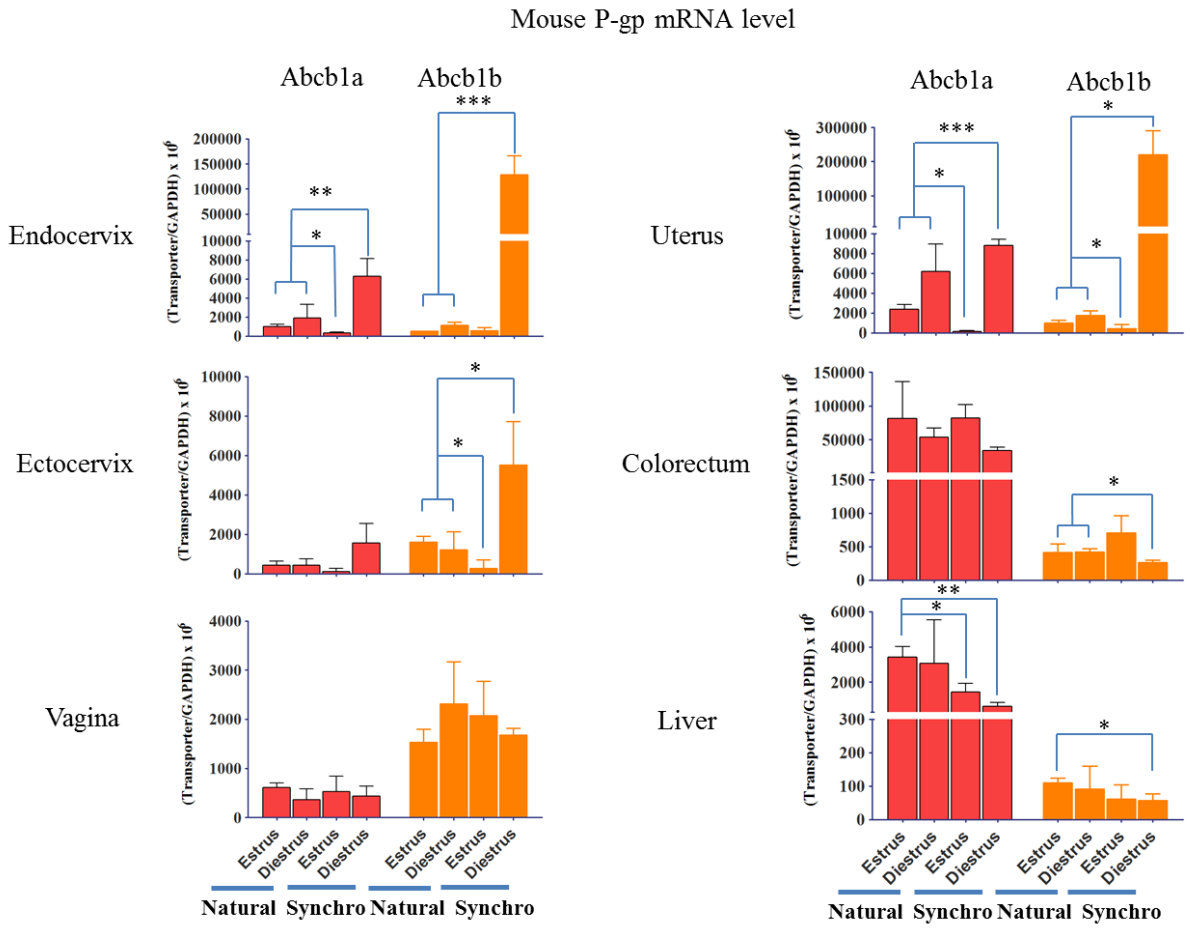


Figure 2.9 Effect of estrus cycle or exogenous hormone/contraceptive on the mRNA expression of P-gp in mouse tissues.

P-gp mRNA level was examined in mouse tissues collected under the following 4 conditions: estrus and diestrus stages during the natural estrous cycle, synchronized estrus after PMSG treatment, and synchronized diestrus after Depo-Provera treatment. Mouse P-gp consists of two isoforms, Abcb1a and Abcb1b. Therefore, the mRNA levels of both isoforms were shown to reflect mouse P-gp expression. The expression in endocervix, ectocervix, vagina, uterus, colorectum and liver were examined in tissues from 3-5 mice. For each mouse, all the 6 kinds of tissues were collected. For mouse uterus and liver, small pieces (30-50 mg) cut from tissue samples were used for RNA extraction and RT-PCR analysis. For mouse endocervix, ectocervix, vagina and colorectum, whole tissue samples were used for analysis. The threshold cycle numbers (Ct) of transporters and Gapdh of each sample were measured in triplicate, and the average Ct was used to reflect the Ct of transporter or Gapdh in this sample. The Gapdh-normalized transporter levels (generated using $2^{-\Delta Ct}$ method) in different tissues were plotted in the figure shown above. The data shown represent mean ± standard deviation of all samples in a given group. Statistical comparison of transporter levels between the 4 conditions was conducted

using one way analysis of variance (ANOVA). * $p<0.05$; ** $p<0.01$, *** $p<0.001$.

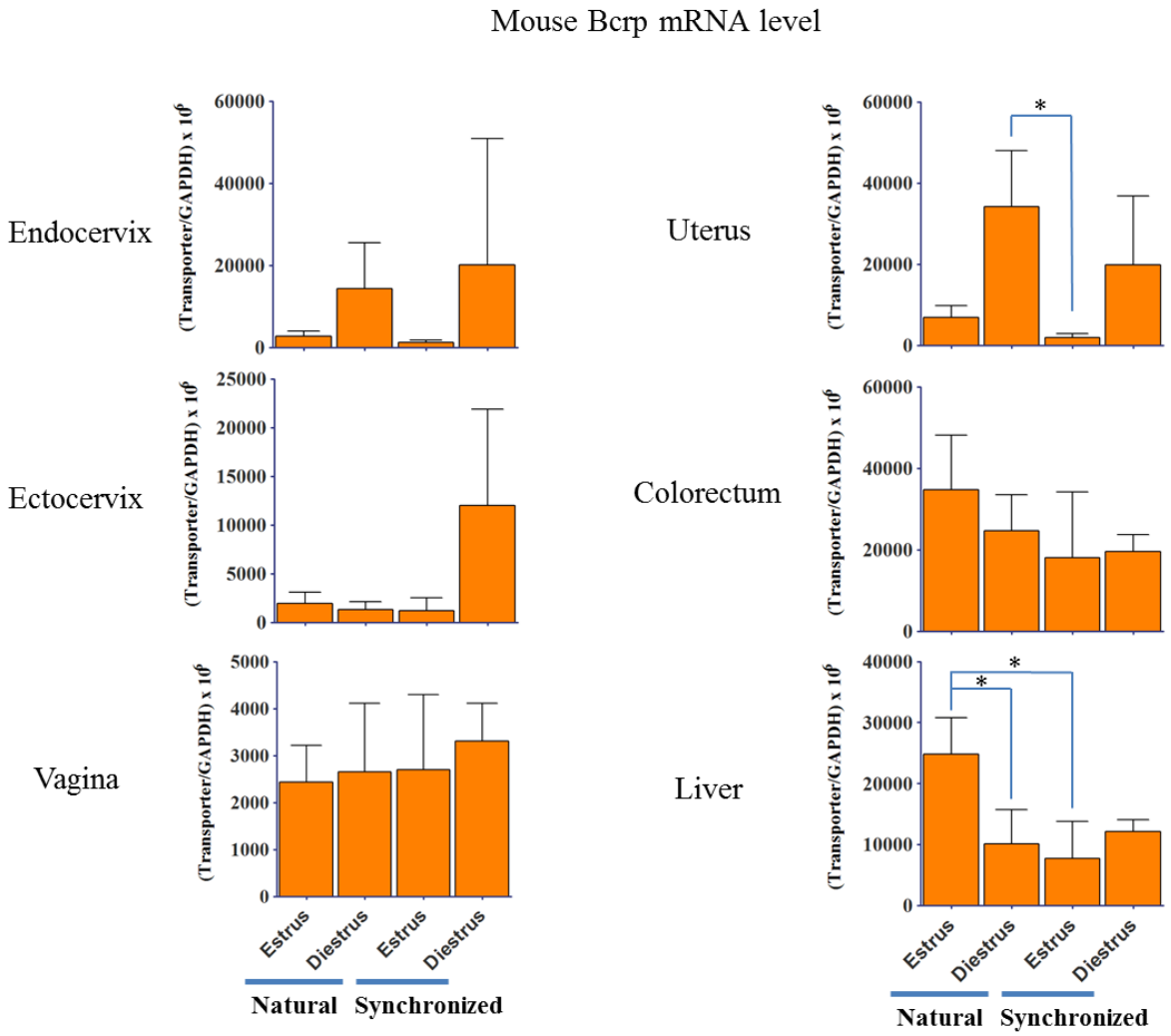


Figure 2.10 Effect of estrus cycle or exogenous hormone/contraceptive on the mRNA expression of Bcrp in mouse tissues.
Bcrp mRNA level was examined in mouse tissues collected under 4 conditions as described in Figure 2.09. Tissue collection and data analysis were performed in the same way as described in Figure 2.09. Data shown represent mean ± standard deviation of all samples in a given group. * $p<0.05$.

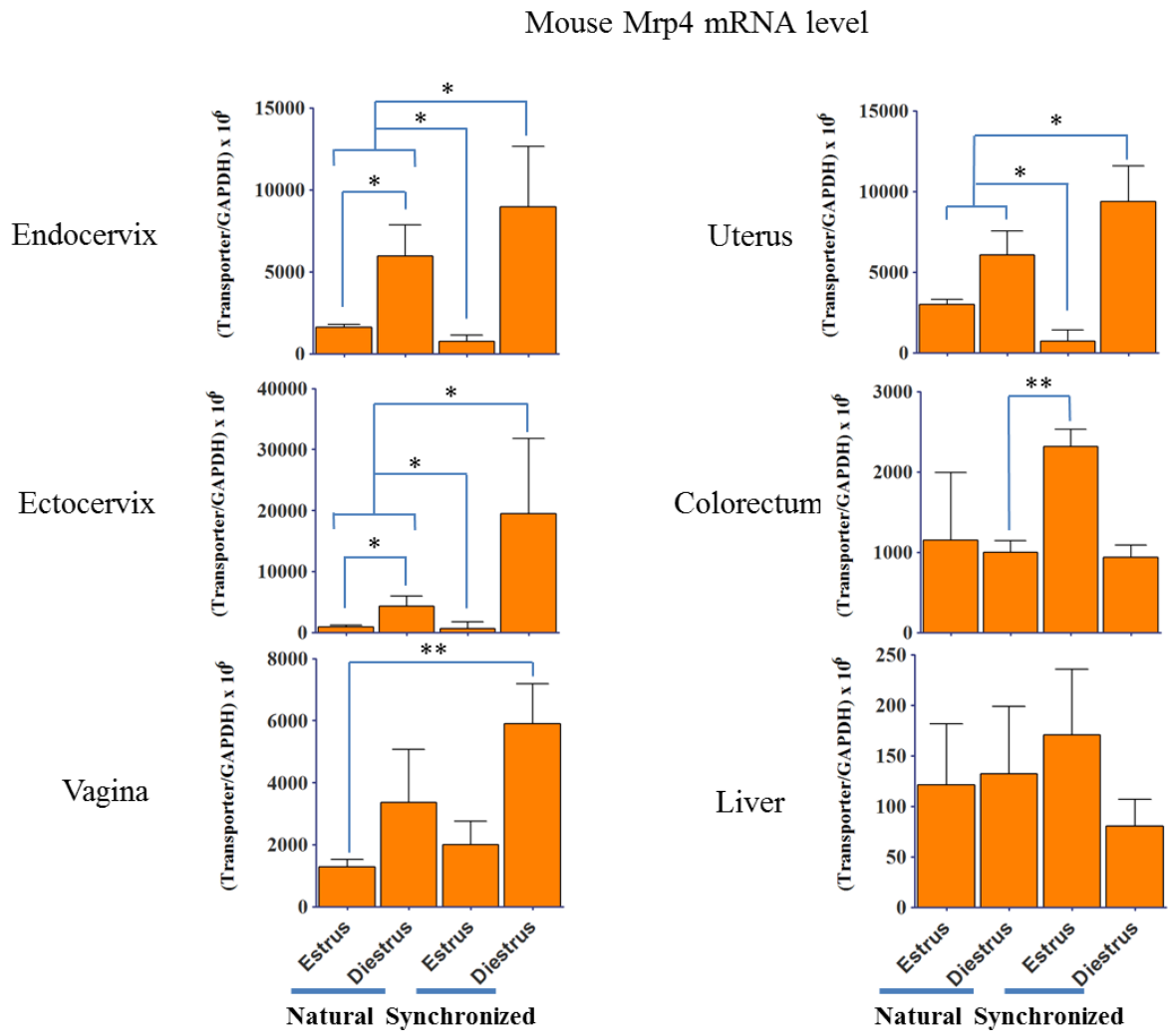


Figure 2.11 Effect of estrus cycle or exogenous hormone/contraceptive on the mRNA expression of Mrp4 in mouse tissues.
Mrp4 mRNA level was examined in mouse tissues collected under 4 conditions as described in Figure 2.09. Tissue collection and data analysis were performed in the same way as described in Figure 2.09. Data shown represent mean ± standard deviation of all samples in a given group. * $p<0.05$; ** $p<0.01$.

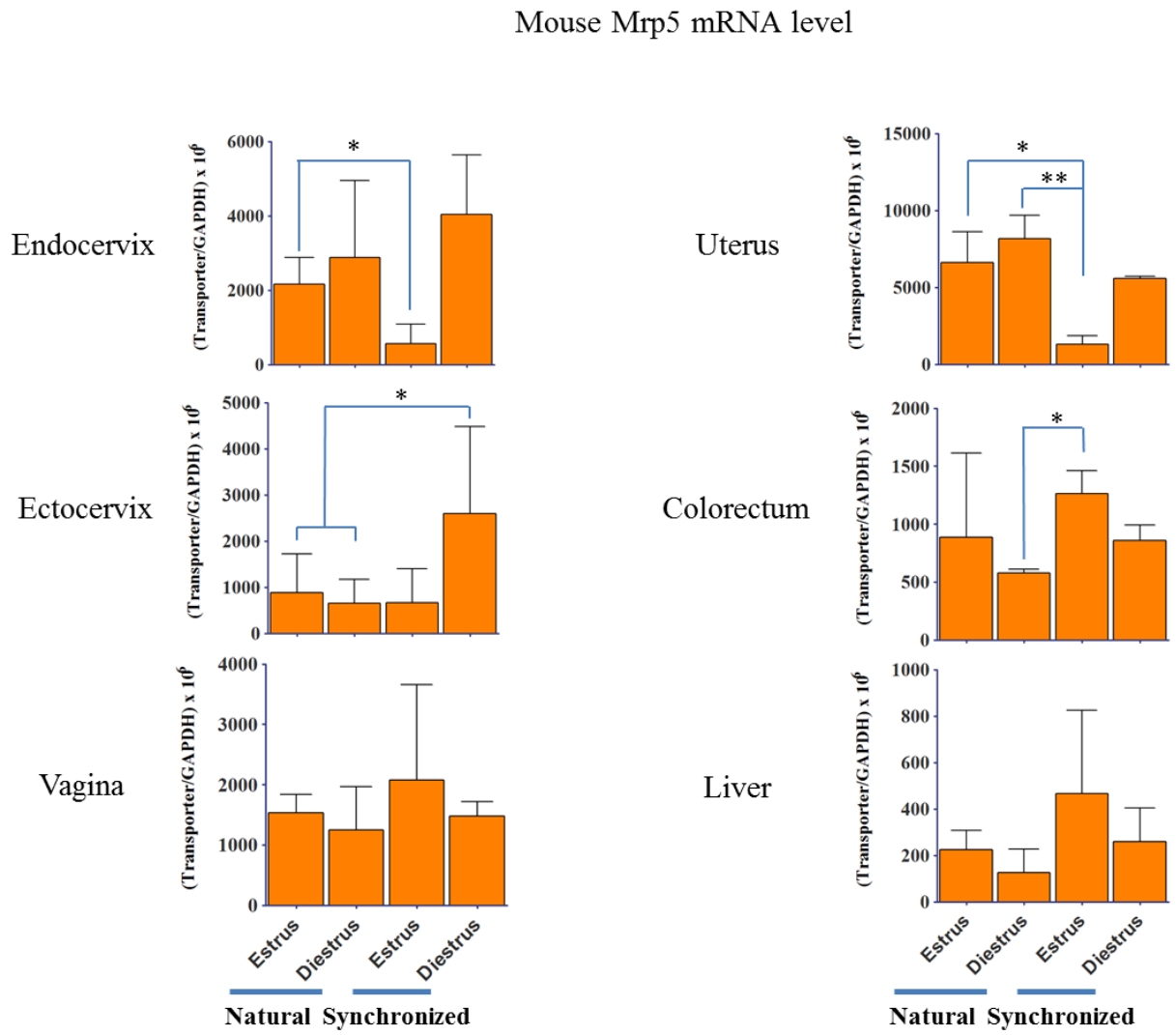


Figure 2.12 Effect of estrus cycle or exogenous hormone/contraceptive on the mRNA expression of Mrp5 in mouse tissues.
Mrp5 mRNA level was examined in mouse tissues collected under 4 conditions as described in Figure 2.09. Tissue collection and data analysis were performed in the same way as described in Figure 2.09. Data shown represent mean ± standard deviation of all samples in a given group. * $p<0.05$; ** $p<0.01$.

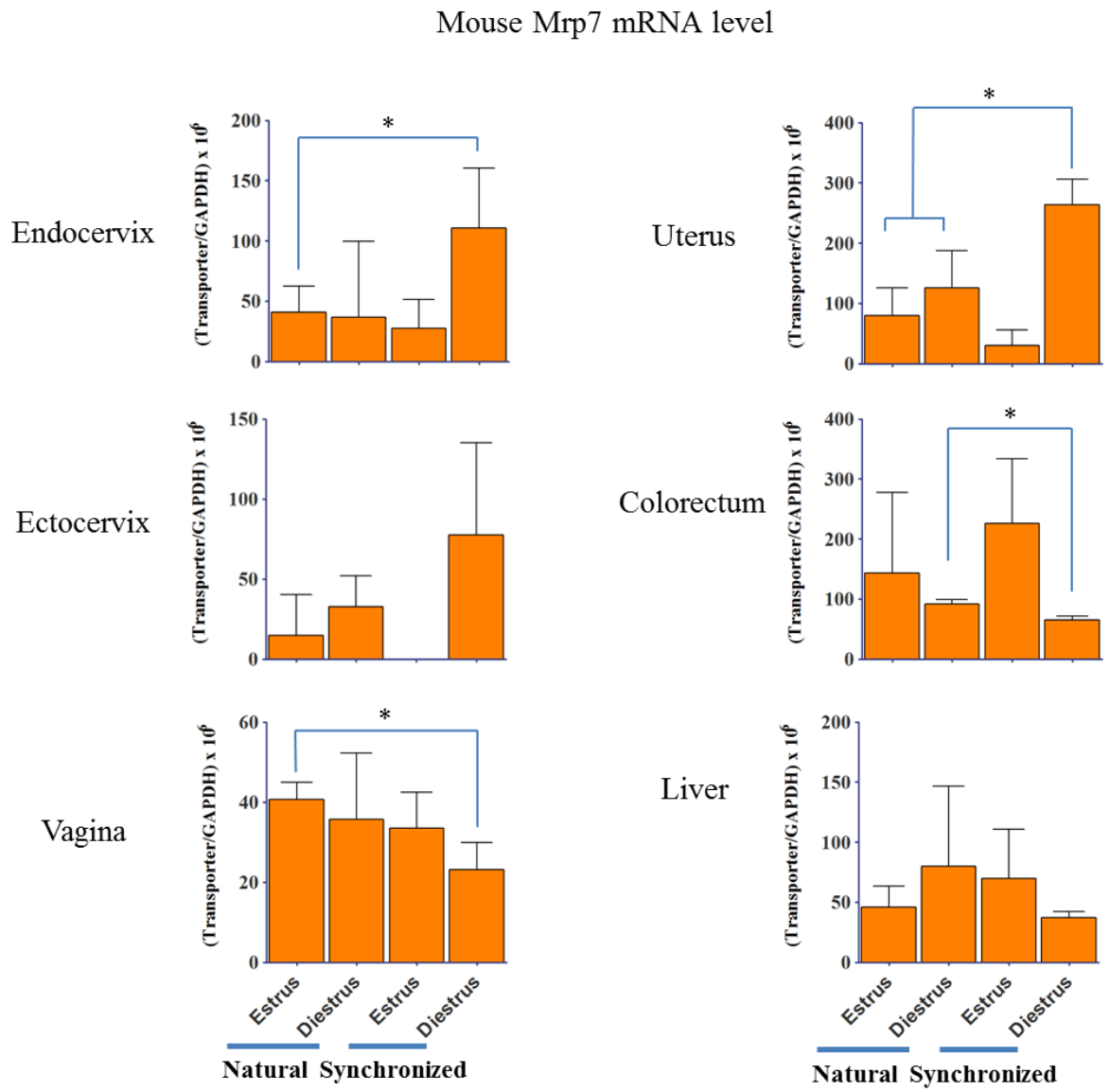


Figure 2.13 Effect of estrus cycle or exogenous hormone/contraceptive on the mRNA expression of Mrp7 in mouse tissues.
Mrp7 mRNA level was examined in mouse tissues collected under 4 conditions as described in Figure 2.09. Tissue collection and data analysis were performed in the same way as described in Figure 2.09. Data shown represent mean ± standard deviation of all samples in a given group. * $p<0.05$.

2.3.6 Real-time RT-PCR examination of mRNA levels of a select panel of transporters in cell lines derived from human cervicovaginal tissues and immune system

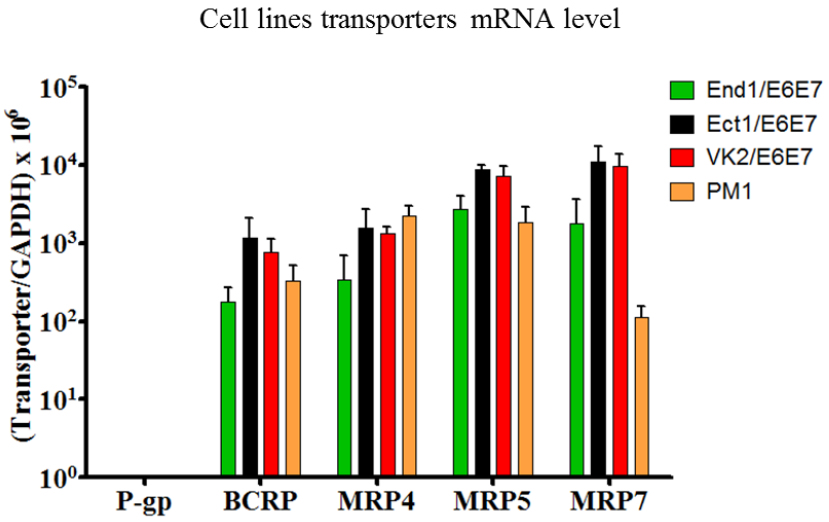


Figure 2.14 Real-time RT-PCR analysis of a select panel of transporters in cell lines derived from human cervicovaginal tissues and T cells.
Three epithelial cell lines derived from human endocervix (End1/E6E7), ectocervix (Ect1/E6E7) and vagina (VK2/E6E7), and a T cell line (PM1) were cultured under basal conditions as described in Materials and Methods above. Cells were collected when 60-90% confluency was reached for the epithelial cells, or good growing condition was maintained for the PM1 cells. The collected cells were then subjected to RNA extraction and RT-PCR, and GAPDH-normalized transporter mRNA levels were plotted. Data represent mean ± SD generated from at least three wells (End1/E6E7, Ect1/E6E7, VK2/E6E7) or three flasks (PM1).

The same panel of efflux transporters were tested in cell lines derived from human endocervical epithelium (End1/E6E7), ectocervical epithelium (Ect1/E6E7), vaginal epithelium (VK2/E6E7), and human T cells (PM1). P-gp was not detectable in all the four cell lines, BCRP, MRP4, MRP5, and MRP7 can be detected in all the cell lines but their expression levels were lower compared to the tissue levels found in human and animal models. There is no significant difference in transporter level between different epithelial cell lines. In addition, when comparing

transporter levels between cervicovaginal epithelial cell lines and the PM1 T cell line, MRP7 was expressed at much lower level in PM1 cells. OAT1 and OAT3 were not detectable in all the cell lines, which is consistent with the observations that these two uptake transporters could be detected by RT-PCR in the cervicovaginal tissues of human, macaque, rabbit and mouse.

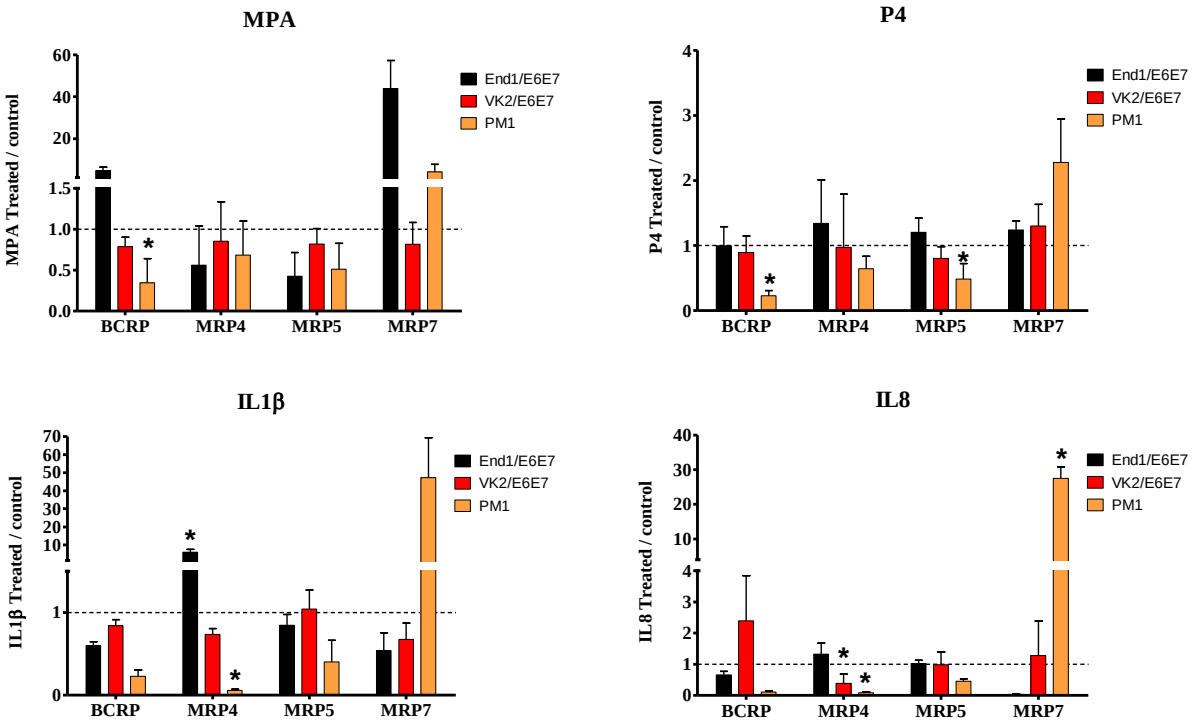


Figure 2.15 Effect of contraceptives and inflammation-related cytokines on the transporter mRNA expression in cervicovaginal and T cell lines.
Two epithelial cell lines (End1/E6E7, VK2/E6E7) were grown on plate, and a T cell line (PM1) was grown in flask. Cells were treated with contraceptives (MPA, P4), or cytokines (IL1β or IL8), or vehicle solution used to dissolve contraceptives/cytokines, for 48 hours. Then, cells were collected and subjected to RT-PCR analysis of the mRNA levels of BCRP, MRP4, MRP5, and MRP7. For each treatment, data were generated from 3-5 wells or flasks. For each transporter examined, the GAPDH-normalized transporter levels (generated using the $2^{-\Delta Ct}$ method) in contraceptive- and cytokine-treated cells were compared to the average GAPDH-normalized transporter level in vehicle-treated cells. These fold changes relative to vehicle-treated controls were expressed as mean ± SD. P-gp was not detectable in End1/E6E7, VK2/E6E7, and PM1 cell lines, under all treatments. MPA, medroxyprogesterone acetate, the active pharmaceutical

ingredient of Depo-Provera. P4, Progesterone. *, $p<0.05$ compared to the vehicle-treated control.

To evaluate the effect of contraceptives and cytokines on transporter mRNA expression in cervicovaginal tissue and immune cells, the End1/E6E7, VK2/E6E7, and PM1 cells were incubated with MPA, P4, IL1β, or IL8 for 48 hours (Since there is no difference between End1/E6E7, Ect1/E6E7 and VK2/E6E7 in transporter mRNA levels, only End1/E6E7 and VK2/E6E7 were tested in this study to represent the single-layer columnar epithelial cells and multi-layer squamous epithelial cells, respectively). The impacts of these treatments were dependent on the type of cell and type of transporter, as shown in Figure 2.15. The general tendency is that the four factors appeared to regulate epithelial cell lines (End1/E6E7, VK2/E6E7) and immune cell line toward different directions.

2.3.7 Multi-species comparison in mRNA levels of highly expressed and most relevant transporters

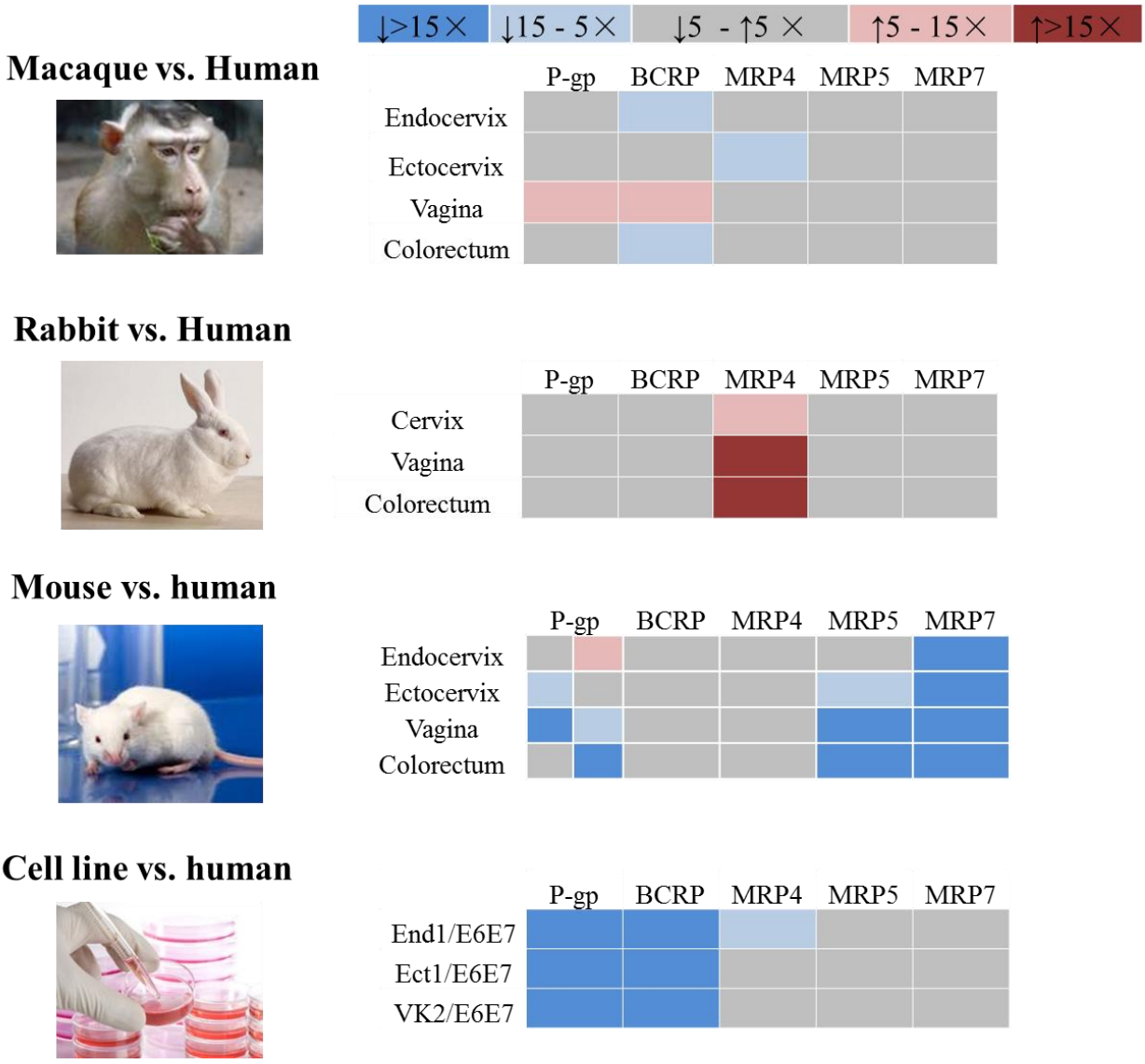


Figure 2.16 Multi-species comparison in mRNA levels of select transporters.

The Gapdh-normalized transporter mRNA levels in animal tissues and cell lines were normalized to the GAPDH-normalized transporter mRNA levels in corresponding human tissues, and the ratios were expressed using different colors indicating different degrees of similarity. Grey color, 5 fold lower or higher than the human tissue level; light blue and light red, 5-15 fold lower or higher; dark blue and dark red, more than 15 fold lower or higher. Human data were shown in Figure 2.4. Pigtailed macaque data were shown in Figure 2.5. New Zealand White rabbit data were shown in Figure 2.6. The mouse data used here for comparison was from Depo-Provera synchronized mice, as shown in Figures 2.9 to 2.13. This synchronized mouse model is used by researchers in the field of vaginal microbicides to evaluate the safety of vaginal products.[198-200] Mouse P-gp transporter consists of two functional isoforms, Abcb1a and Abcb1b. Therefore, the average level of each isoform was compared to average human P-gp level, and the results of both isoforms were listed together to indicate the similarity in P-gp levels between mouse and human. The three cell lines used for comparison are derived from the epithelial cells of human

endocervix (End1/E6E7), ectocervix (Ect1/E6E7), and vagina (VK2/E6E7).

The transporter mRNA levels (using GAPDH as internal control) were compared across different species/models. The purpose of this comparison is to inform model selection for future studies of transporter function, judging from the mRNA expression data. It might be not practical to find a model that has identical expression pattern of multiple transporters, but such comparisons will help the researchers make informed decision and will help correlating pharmacokinetic/pharmacodynamics data between human subjects and preclinical models.

As shown in Figure 2.16, pigtailed macaque appeared to be the model with highest similarity to human, in terms of cervicovaginal and colorectal transporter expression pattern. Rabbit Mrp4 levels was more than 15-fold higher than human levels, but the difference for other transporters is within ± 5 fold. The levels of P-gp, BCRP, Mrp4 and Mrp5 in Depo-Provera synchronized mouse tissues were similar to human's in certain tissue segments, but the difference in Mrp7 was higher than 15 fold for all mouse tissues examined. When comparing the three cervicovaginal epithelial cell lines to corresponding human tissues, the differences were larger than 15 fold for P-gp and BCRP, but the differences were within ± 5 fold for MRP4, 5, 7, except that MRP4 level in End1/E6E7 cells was more than 5 times lower than the MRP4 level in human endocervical tissue. The absence of OAT1 and OAT3 in all the tissues and cells examined suggested that these two uptake transporters were not likely to play a functional role in drug pharmacokinetics in the cervicovaginal and colorectal tissues.

2.3.8 Real-time RT-PCR examination of mRNA levels of nuclear receptors (NRs) in human, macaque, and mouse tissues

The mRNA levels of 23 NRs were examined in the cervicovaginal and colorectal tissues of human, macaque, and Depo-Provera-synchronized mice (Figures 2.17 to 2.19). The purpose of this examination was to provide clues for the mechanism study of transporter regulation, and to evaluate the utility of different preclinical models in the study of NR-mediated regulation in cervicovaginal and colorectal tracts. The determination of the panel of NRs was based on their reported interactions with drug transporters in other tissues such as liver.

The GAPDH-normalized NR mRNA levels were used to compare the expression of each NR across different tissues, and between different NRs in the same type of tissue. Since liver has abundant expression of many NRs, the GAPDH-normalized levels were further divided by the average of liver levels in each species, and results were shown in the tables associated with Figures 2.17 to 2.19.

In human endocervix, ectocervix, and vagina, many NRs were found to express at moderate to high levels. These NRs included VDR, PPAR-α, PPAR-β, PPAR-γ, ER-α, PR, AR, GR, MR, RAR-α, RAR-β, RAR-γ, RXR-α, RXR-β, RXR-γ, ROR-α, ROR-β, Nrf2, and AhR. The mRNA levels of these NRs were above 1/1000 of GPADH levels in corresponding tissues. The NRs with highest GAPDH-normalized mRNA levels (around 1/10 GAPDH level or higher) were ER-α, PR, AR, GR, Nrf2, and AhR, all of which could respond to sex steroid hormones or oxidative stress. On the contrary, PXR, CAR, ER-β and RAR-γ were expressed at low levels (<1/1000 of GAPDH level) in human cervicovaginal tissues. The levels of VDR, PPAR-γ, MR, RAR-β,

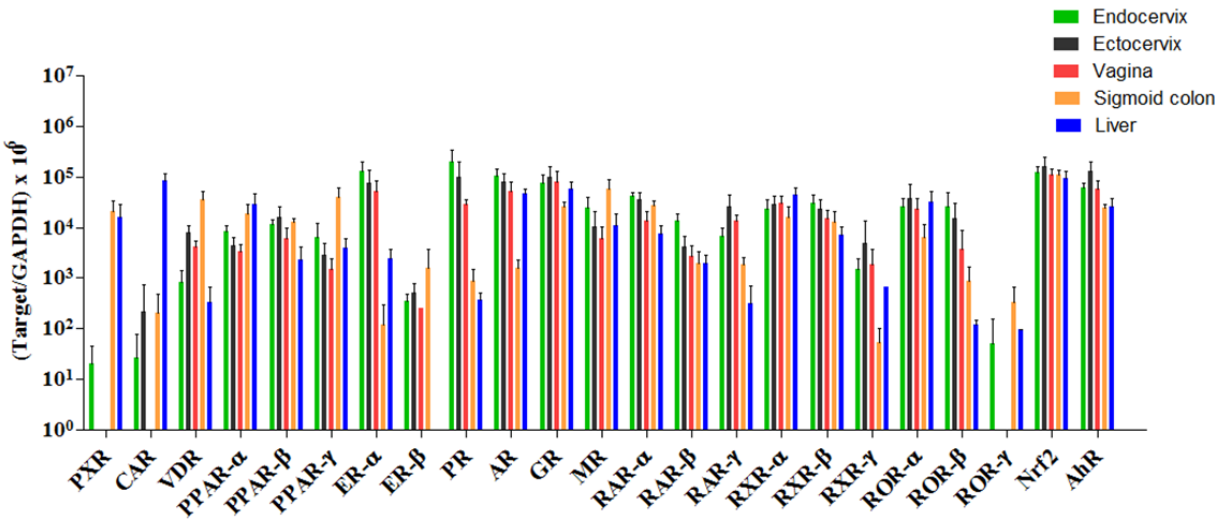
RAR-γ, ROR-β appeared to differ between endocervix, ectocervix, and vagina. RAR-β in endocervix was significantly higher than that in ectocervix and vagina (p <0.001). No significant difference was observed for other NRs between the three segments of human female lower genital tract. The moderately and highly expressed NRs in lower genital tract may play a role in regulating the mRNA expression of transporters and other downstream genes in human cervicovaginal tissues. In addition, the difference in expression levels between endocervix, ectocervix and vagina may lead to differential regulation by the same NR in different segments of lower genital tract

In human colorectal tissue, the expression profile of many NRs was found to be comparable to that of cervicovaginal tissues, but some NRs demonstrated differential expression levels. Similar to cervicovaginal tissues, VDR, PPAR-α, PPAR-β, PPAR-γ, AR, GR, MR, RAR-α, RAR-β, RAR-γ, RXR-α, RXR-β, ROR-α, Nrf2, and AhR were above 1/1000 of GAPDH level. However, ER-α, PR, RXR-γ, ROR-β, ROR-γ were found be express at levels lower than 1/1000 of GAPDH although they displayed high expression in human cervicovaginal tissues. In addition, PXR and ER-β were expressed at levels higher than 1/1000 of GPADH in human colorectal tissue, which was another difference from the cervicovaginal tissues. Judging from the GAPDH-normalized levels, the most highly expressed NRs were MR and Nrf2, and the least expressed NRs were CAR and ROR-γ, in human colorectal tissue. With respect to the statistical difference between colorectal tissue and cervicovaginal tissues, PXR, VDR, PPAR-γ, and MR levels in colorectal tissue was significantly higher than that in cervicovaginal tissues (p <0.05, p <0.001, p <0.001, p <0.05, respectively); On the other hand, ER-α and PR, RAR-β, ROR-β levels in colorectal tissue was significantly lower than their levels of endocervix (p <0.01, p <0.01, p <0.01, p <0.05,

respectively), RAR-γ level in colorectal tissue was significantly lower than that in human ectocervix (p <0.01), AR level in colorectal tissue was significantly lower than its levels in endocervix and ectocervix (p <0.01). The moderately and highly expressed NRs in human colorectal tissue may play a role in regulating the mRNA expression of transporters and other downstream genes in this tissue In addition, the difference in expression levels between colorectal and cervicovaginal tissues may lead to differential regulation by the same NR activator between these tissue sites.

Human liver has abundant expression of many NRs, and cervicovaginal and colorectal tissues displayed comparable or even higher levels for some of the NRs highly expressed in liver. Judging from the GAPDH-normalized mRNA levels, PXR, CAR, PPAR-α, PPAR-β, PPAR-γ, ER-α, AR, GR, MR, RAR-α, RAR-β, RXR-α, RXR-β, ROR-α, Nrf2, and AhR were expressed at levels higher than 1/1000 of GAPDH. CAR and Nrf2 levels were found to be highest (around 1/10 of GAPDH). These NRs can be activated by therapeutic drugs (e.g. PXR), hormones (e.g. ER-α), nutrients (e.g. RAR-α), and oxidative stress (e.g. Nrf2). As shown in the table associated with Figure 2.17, some of the NRs that showed moderate to high expression in human liver demonstrated comparable (p > 0.05) or even higher (p < 0.05) levels in cervicovaginal and/or colorectal tissues, including PXR (colorectal tissue, p > 0.05), PPAR-α (colorectal tissue, p > 0.05), PPAR-β (ectocervix, p < 0.01; colorectal tissue, p < 0.05), PPAR-γ (colorectal tissue, p < 0.001), ER-α (endocervix, p < 0.001; ectocervix, p < 0.05), AR (endocervix, p < 0.05), MR (colorectal tissue, p < 0.001), RAR-α (endocervix and ectocervix, p < 0.001; colorectal tissue, p < 0.05), RAR-β (endocervix, p < 0.001), RXR-β (endocervix, p < 0.01), and AhR (ectocervix, p < 0.001). To summarize, a number of NRs that had moderate to high expression in human liver

displayed comparable or higher expression levels in human cervicovaginal and colorectal tissues. This implicated that these NRs may play a role in regulating their downstream genes in cervicovaginal and colorectal tissues.



NR tissue levels relative to human liver level				
Nuclear receptors	**Endocervix**	**Ectocervix**	**Vagina**	**Colorectal tissue**
PXR	<0.01	<0.01	<0.01	1.30±0.78
CAR	<0.01	<0.01	<0.01	<0.01
VDR	2.50±1.72	23.00±9.78	12.00±3.83	102.87±53.69
PPAR-α	0.30±0.08	0.15±0.07	0.12±0.04	0.65±0.39
PPAR-β	4.94±1.47	6.92±4.50	2.71±1.57	5.69±0.81
PPAR-γ	1.61±1.53	0.72±0.49	0.37±0.24	10.03±5.31
ER-α	54.81±29.14	32.00±25.26	22.06±12.08	0.37±0.34
ER-β	N/A	N/A	N/A	N/A
PR	6425.95±4356.14	3149.10±3072.62	761.87±431.85	23.82±24.03
GR	1.30±0.57	1.64±0.99	1.37±0.77	0.44±0.10
AR	2.27±0.89	1.77±0.84	1.12±0.64	0.04±0.01
MR	2.24±1.32	0.95±0.97	0.56±0.38	5.45±2.61
RAR-α	5.51±1.07	4.53±1.99	1.81±0.97	3.62±0.73
RAR-β	6.91±2.62	2.10±1.25	1.39±0.87	0.99±0.67
RAR-γ	23.97±10.43	89.46±68.96	46.32±14.62	6.67±2.39
RXR-α	0.53±0.28	0.67±0.30	0.69±0.27	0.45±0.13
RXR-β	4.27±1.80	3.13±1.73	2.07±0.89	2.19±0.58
RXR-γ	42.18±24.32	138.92±247.23	53.03±49.75	<0.01
ROR-α	0.81±0.34	1.17±1.03	0.72±0.44	0.31±0.16
ROR-β	1034.84±924.14	623.62±622.90	124.87±191.63	26.64±38.78
ROR-γ	N/A	N/A	N/A	N/A
Nrf2	1.33±0.42	1.77±0.84	1.17±0.35	1.16±0.30
AhR	2.49±0.71	5.29±2.84	2.33±1.07	1.07±0.48

Figure 2.17 Real-time RT-PCR analysis of nuclear receptors (NRs) in human cervicovaginal and colorectal tissues.
The NR mRNA expression in human endocervix, ectocervix, vagina, sigmoid colon and liver were examined in tissues from 4-6 donors (Endocervix, 4 donors; ectocervix, 6 donors; vagina, 5 donors; sigmoid colon, 5 donors; liver, 6 donors). The GAPDH-normalized NR levels (generated using $2^{-\Delta Ct}$ method) in different tissues were plotted in the figure shown above. the GAPDH-normalized NR levels in genital tract and colorectal tissues relative to the GAPDH-normalized NR levels in human liver were summarized in the table. The data shown represent mean ± standard deviation. N/A, the NR cannot be detected in liver, so that the normalization of cervicovaginal/colorectal level to liver level cannot be performed.

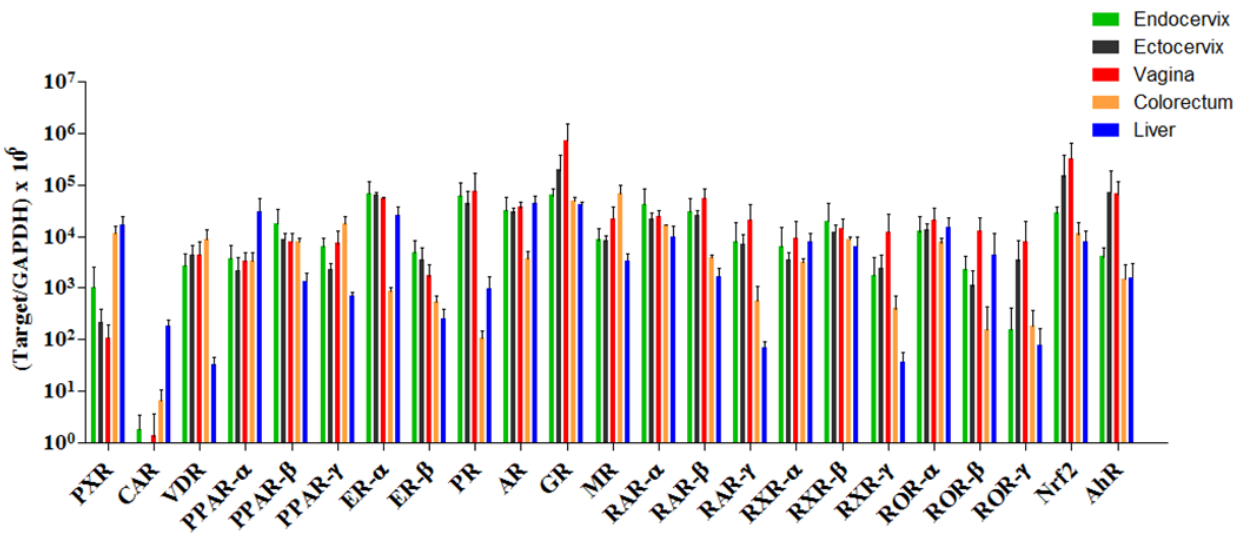
The mRNA expression profile of NRs in macaque cervicovaginal and colorectal tissues were generally similar to that of corresponding human tissues Figure 2.18. The NRs that can interact with steroid hormones were expressed at high levels compared to macaque liver and colorectum. Similarly, the xenobiotic-binding NRs such as PXR was almost undetectable in macaque cervicovaginal tissues but was expressed in macaque colorectum at the levels similar to liver.

In macaque endocervix, ectocervix, and vagina, many NRs were found to express at moderate to high levels. VDR, PPAR-α, PPAR-β, PPAR-γ, ER-α, PR, AR, GR, MR, RAR-α, RAR-β, RAR-γ, RXR-α, RXR-β, RXR-γ, ROR-α, ROR-β, Nrf2, and AhR were found to express at levels above 1/1000 of macaque Gapdh. The moderate to high expression of these NRs was similar to the expression pattern of these NRs in human tissues (Figure 2.17). Similar to human tissues, ER-α, PR, AR, GR, Nrf2, and AhR were among the most highly expressed NRs in macaque cervicovaginal tissues. PXR, CAR, ER-β and RAR-γ were expressed at low level (<1/1000 of Gapdh level) in human cervicovaginal tissues. For these NRs, the levels CAR in macaque cervicovaginal tissues were undetectable, but the levels of PXR, ER-β, and RAR-γ were positively detected and even higher than 1/1000 of Gapdh (Figure 2.18). The levels of PXR, PPAR-γ, ER-β, GR, MR, RXR-γ, ROR-β, ROR-γ, Nrf2, and AhR appeared to differ between

endocervix, ectocervix, and vagina. However, no statistical difference has been observed for these NRs between the three segments of macaque cervicovaginal tract, possibly due to limited number of macaque tissues used in this study (n=3 for each kind of tissue). The moderately and highly expressed NRs in macaque cervicovaginal tissues could respond to therapeutic drugs (e.g. PXR), steroid hormones (e.g. ER-α), nutrients (e.g. RAR-α), and oxidative stress (e.g. Nrf2). Although the absolute levels of these NRs are not identical to those in human cervicovaginal tissues, the NR expression pattern was generally comparable between macaque and human cervicovaginal tissues.

In macaque colorectal tissue, the expression profile of many NRs was found to be comparable to that of human colorectal tissue. The moderately to highly expressed NRs in human tissue, including PXR, VDR, PPAR-α, PPAR-β, PPAR-γ, AR, GR, MR, RAR-α, RAR-β, RXR-α, RXR-β, ROR-α, Nrf2, and AhR, were also found to be expressed at moderate to high levels in macaque colorectal tissue (1/1000 to 1/10 of Gapdh, Figure 2.18). ER-β and RAR-γ were expressed at slightly lower levels compared to human colorectal tissue, but were still around 1/1000 of Gapdh. The lowly expressed or undetectable NRs (<1/1000 of Gapdh) in human colorectal tissue, including ER-α, PR, RXR-γ, ROR-β, ROR-γ, were expressed at low levels in macaque colorectal tissue. Compared to macaque cervicovaginal tissues; the colorectal PPAR-γ level was significantly higher than its ectocervical level (p <0.05); the colorectal ER-α level was significantly lower than its endocervical (p <0.05) and ectocervical (p <0.05) levels; the colorectal MR level was significantly higher than its endocervical (p <0.05) and ectocervical (p <0.05) levels. Overall, the expression pattern of NRs in macaque colorectal tissue was generally similar to that of human colorectal tissue.

Similar to human liver, the macaque liver also demonstrated abundant expression of many NRs. In addition, macaque cervicovaginal and colorectal tissues displayed comparable or even higher levels for some of the NRs highly expressed in macaque liver. Judging from the Gapdh-normalized mRNA levels, PXR, PPAR-α, PPAR-β, ER-α, AR, GR, MR, RAR-α, RAR-β, RXR-α, RXR-β, ROR-α, ROR-β, Nrf2, and AhR were expressed at levels higher than 1/1000 of Gapdh in macaque liver. The Gapdh-normalized NR levels in macaque cervicovaginal and colorectal tissues were divided by their levels in macaque liver, and the ratios were shown in the table associated with Figure 2.18 for comparison. Some of the NRs that showed moderate to high expression in macaque liver demonstrated comparable ($p > 0.05$) or even higher ($p < 0.05$) levels in cervicovaginal and/or colorectal tissues, including PPAR-β (colorectal tissue, $p < 0.01$), MR (colorectal tissue, $p < 0.01$). The cervicovaginal or colorectal tissues levels of several other NRs such as Nrf2 and AhR also appeared to be higher than their levels in liver, however the statistical significance was not achieved, probably due to limited number of tissues used in this study (n=3 for each type of tissue). To summarize, a number of NRs that had moderate to high level of mRNA expression in macaque liver demonstrated comparable or higher levels in macaque cervicovaginal and colorectal tissues. This was similar to the comparison between human tissues (Figure 2.17), and implicated that multiple NRs may play a functional role in regulating downstream genes in macaque cervicovaginal and colorectal tissues.



NR tissue levels relative to macaque liver level

Nuclear receptors	Endocervix	Ectocervix	Vagina	Colorectum
PXR	0.06 ± 0.09	0.01 ± 0.01	<0.01	0.71 ± 0.23
CAR	<0.01	<0.01	<0.01	0.04 ± 0.02
VDR	82.83 ± 63.65	134.63 ± 68.18	137.18 ± 102.83	267.90 ± 150.47
PPAR-α	0.12 ± 0.10	0.07 ± 0.05	0.11 ± 0.05	0.11 ± 0.05
PPAR-β	13.20 ± 11.82	6.41 ± 2.11	5.97 ± 2.51	5.88 ± 1.09
PPAR-γ	8.82 ± 4.15	3.18 ± 0.95	10.21 ± 7.65	24.51 ± 10.31
ER-α	2.61 ± 1.82	2.53 ± 0.21	2.09 ± 0.08	0.03 ± 0.01
ER-β	18.69 ± 14.53	13.90 ± 9.11	6.62 ± 4.40	2.09 ± 0.57
PR	63.44 ± 48.91	44.35 ± 33.87	77.62 ± 95.48	0.11 ± 0.04
GR	1.53 ± 0.55	4.75 ± 4.67	17.51 ± 19.31	1.22 ± 0.21
AR	0.73 ± 0.58	0.68 ± 0.14	0.84 ± 0.26	0.09 ± 0.03
MR	2.57 ± 1.63	2.49 ± 0.66	6.66 ± 4.63	19.81 ± 9.30
RAR-α	4.25 ± 4.20	2.25 ± 0.71	2.50 ± 0.85	1.69 ± 0.01
RAR-β	18.33 ± 15.55	15.87 ± 3.88	33.01 ± 19.30	2.42 ± 0.27
RAR-γ	116.22 ± 152.01	102.85 ± 54.61	296.28 ± 303.58	8.25 ± 7.85
RXR-α	0.82 ± 1.07	0.44 ± 0.16	1.20 ± 1.30	0.41 ± 0.08
RXR-β	3.04 ± 3.88	1.91 ± 0.73	2.23 ± 1.25	1.33 ± 0.22
RXR-γ	47.45 ± 56.50	64.60 ± 56.10	329.63 ± 392.97	10.73 ± 8.71
ROR-α	0.82 ± 0.78	0.88 ± 0.29	1.37 ± 1.04	0.51 ± 0.10
ROR-β	0.53 ± 0.42	0.26 ± 0.24	2.89 ± 2.55	0.04 ± 0.06
ROR-γ	1.98 ± 3.17	43.81 ± 64.78	100.00 ± 157.07	2.30 ± 2.45
Nrf2	3.57 ± 1.16	18.60 ± 28.00	40.76 ± 41.29	1.40 ± 0.91
AhR	2.97 ± 1.85	45.09 ± 73.94	41.91 ± 32.31	0.96 ± 0.80

Figure 2.18 Real-time RT-PCR analysis of nuclear receptors (NRs) in macaque cervicovaginal and colorectal tissues.

The NR mRNA expression in macaque endocervix, ectocervix, vagina, colorectum and liver were examined in tissues collected from 3 macaques. For each macaque, all the 5 kinds of tissues were collected. The Gapdh-normalized NR levels (generated using $2^{-\Delta Ct}$ method) in different tissues were plotted in the figure shown above. The Gapdh-normalized NR levels in genital tract and colorectal tissues relative to the Gapdh-normalized NR levels in macaque liver were

summarized in the table. The data shown represent mean ± standard deviation.

The NR expression profile in mouse cervicovaginal and colorectal tissues were generally comparable to that in human and macaque tissues (Figure 2.19). The hormone-binding NRs were expressed at much higher level in mouse cervix and vagina compared to mouse colorectum or liver; while the xenobiotic binding NRs such as PXR was expressed at much higher level in mouse colorectum compared to cervicovaginal tissues.

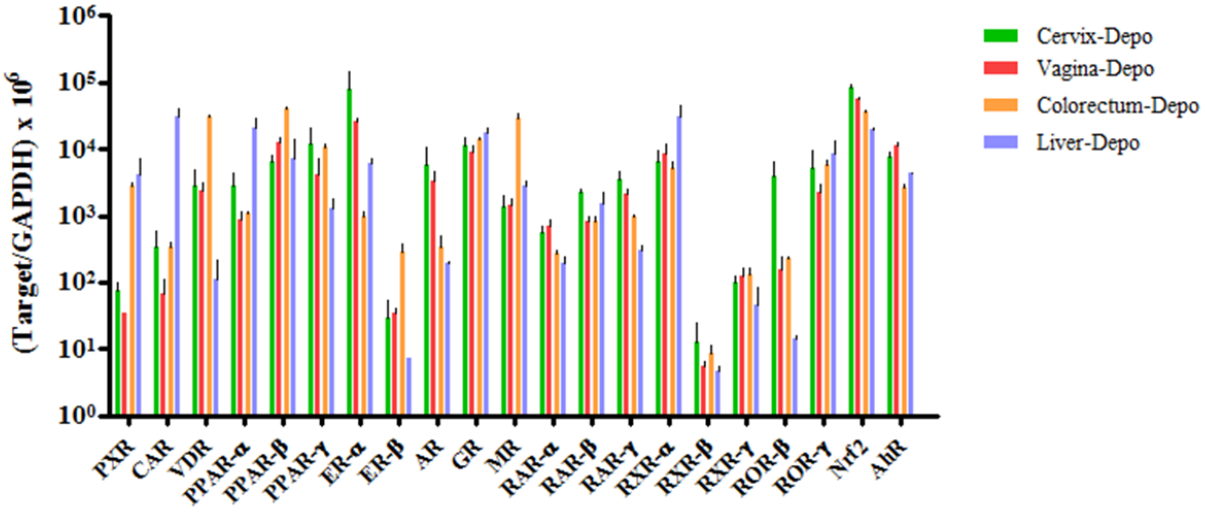
In the examination of NR expression in mouse cervicovaginal tissues, the endocervix and ectocervix were combined for RNA extraction and real-time RT-PCR (Figure 2.19). In mouse cervix and vagina, many NRs were found to express at moderate to high levels (>1/1000 of mouse Gapdh level), including VDR, PPAR-α, PPAR-β, PPAR-γ, ER-α, AR, GR, MR, RAR-β, RAR-γ, RXR-α, ROR-α, ROR-β, ROR-γ, Nrf2, and AhR. On the other hand, a few NRs such as PXR, CAR, and ER-β were found to be expressed at low levels or undetectable (<1/1000 of mouse Gapdh). This expression pattern of NRs is similar to that in human cervicovaginal tissues (Figure 2.17). Differences in NR expression between mouse cervix and vagina have been observed. RAR-β and Nrf2 levels in mouse cervix were significantly higher than those in vagina (p <0.05 and p <0.01, respectively); AhR level in mouse cervix was significantly lower than that in mouse vagina (p <0.05). Although the absolute mRNA levels of NRs in mouse cervix and vagina were not identical to those in human cervicovaginal tissues, the pattern of expression was generally comparable between mouse and human cervicovaginal tissues.

In mouse colorectum, the expression profile of many NRs was found to be comparable to that of human colorectal tissue. The moderately to highly expressed NRs in human tissue, including

PXR, VDR, PPAR-α, PPAR-β, PPAR-γ, GR, MR, RAR-β, RAR-γ, RXR-α, Nrf2, and AhR were also found to be expressed at moderate to high levels in mouse colorectum (1/1000 to 1/10 of Gapdh, Figure 2.19). ER-α and ROR-γ were expressed at low levels (<1/1000 of human GAPDH) in human colorectum tissue, but was above 1/1000 of mouse Gapdh level in mouse colorectum. Compared to mouse cervix and vagina, mouse colorectal levels of VDR, PPAR-β, ER-β, and MR were significantly higher than their levels in mouse cervix (p <0.001 for all NRs) and vagina (p <0.001 for all NRs); colorectal levels of RAR-α was significantly higher than its level in mouse vagina (p <0.05); colorectal levels of RAR-β and RAR-γ were significantly lower than their levels in mouse cervix (p <0.05 and p <0.01, respectively); colorectal levels of Nrf2 and AhR were significantly lower than their levels in mouse cervix (p <0.001 and p <0.01, respectively) and vagina (p <0.01 and p <0.001, respectively). Mouse colorectal PXR level appeared to be much higher than that in mouse cervix and vagina, however statistical significance was not achieved. The pattern of NR expression in mouse colorectum was generally similar to that in human colorectal tissue.

Similar to human liver, mouse liver also demonstrated abundant expression of many NRs. In addition, mouse cervicovaginal and colorectal tissues displayed comparable or even higher levels for some of the NRs highly expressed in mouse liver. The mouse Gapdh-normalized mRNA levels of PXR, CAR, PPAR-α, PPAR-β, PPAR-γ, ER-α, GR, MR, RAR-β, RXR-α, ROR-γ, Nrf2, and AhR were expressed at levels higher than 1/1000 of Gapdh in mouse liver. Some of these NRs demonstrated comparable (p > 0.05) or even higher (p < 0.05) levels in cervicovaginal and/or colorectal tissues, including PXR (colorectum, p > 0.05), PPAR-β (colorectum, p < 0.001), MR (colorectum, p < 0.001), Nrf2 (cervix, p < 0.001; vagina, p < 0.001; colorectum, p < 0.05),

AhR (cervix, $p < 0.05$; vagina, $p < 0.001$). Overall, a number of NRs that had moderate to high levels of mRNA expression in mouse liver demonstrated comparable or higher levels in mouse cervicovaginal and colorectal tissues. This was similar to the comparison between human tissues (Figure 2.17), and implicated a functional role of NRs in mouse cervicovaginal and colorectal tissues.



NR tissue levels relative to mouse liver level			
Nuclear receptors	**Cervix**	**Vagina**	**Colorectum**
PXR	0.01±0.01	<0.01	0.66±0.09
CAR	0.01±0.01	<0.01	0.01±0.00
VDR	38.39±29.54	32.19±10.17	421.02±18.97
PPAR-α	0.14±0.08	0.04±0.01	0.05±0.00
PPAR-β	0.91±0.19	1.76±0.27	5.53±0.51
PPAR-γ	9.38±6.93	3.13±2.45	8.35±1.15
ER-α	12.60±11.16	4.32±0.52	0.16±0.03
ER-β	N/A	N/A	N/A
GR	0.63±0.21	0.52±0.10	0.81±0.06
AR	30.19±25.15	16.96±6.00	1.69±0.87
MR	0.49±0.25	0.52±0.12	10.73±1.40
RAR-α	2.97±0.73	3.58±1.08	1.40±0.15
RAR-β	1.50±0.19	0.56±0.10	0.55±0.12
RAR-γ	11.77±3.81	7.03±1.31	3.23±0.10
RXR-α	0.20±0.09	0.28±0.10	0.16±0.04
RXR-β	2.69±2.68	1.18±0.17	1.81±0.53
RXR-γ	30.38±7.92	38.78±12.50	41.68±9.99
ROR-α	N/A	N/A	N/A
ROR-β	412.12±280.16	13.94±13.11	24.65±1.20
ROR-γ	0.61±0.56	0.27±0.08	0.70±0.10
Nrf2	4.24±0.51	2.90±0.12	1.84±0.11
AhR	1.82±0.32	2.62±0.39	0.62±0.07

Figure 2.19 Real-time RT-PCR analysis of nuclear receptors (NRs) in cervicovaginal and colorectal tissues of Depo-Provera synchronized mice.

The NR mRNA expression in mouse cervix (containing both endocervix and ectocervix), vagina, colorectum and liver were examined in tissues collected from 3 mice receiving Depo-Provera synchronization. For each mouse, all the 4 kinds of tissues were collected. The Gapdh-normalized NR levels (generated using $2^{-\Delta Ct}$ method) in different tissues were plotted in the figure shown above. The Gapdh-normalized NR levels in genital tract and colorectal tissues relative to the Gapdh-normalized NR levels in mouse liver were summarized in the table. The data shown represent mean ± standard deviation. N/A, the NR cannot be detected in liver, so that the normalization of cervicovaginal/colorectal level to liver level cannot be performed.

2.4 Discussion and Conclusion

There is an urgent need to identify critical determinants of cervicovaginal tissue drug pharmacokinetics and pharmacodynamics (PK/PD) to optimize PrEP strategies.[42,201,202] Efflux transporters and uptake transporters have been reported to pump out or uptake many antiretroviral drugs.[49,60,86] If these transporters are present and functional in the cervicovaginal tissues, they will likely affect the distribution of topically or orally administered drugs to the sites of HIV-1 transmission. Our previous study and the current study in this chapter have examined the mRNA expression of transporters and enzymes in human lower genital tract (ectocervix and vagina) using conventional RT-PCR.[201] While a number of efflux transporters (P-gp, BCRP, MRP4, MRP5, MRP7) were found to be expressed at moderate to high levels compared to liver, only a limited number of uptake transporters such as ENT1 were found to be positively expressed in both epithelium and stroma of ectocervix and vagina.[201] OAT1 and OAT3, the major uptake transporters for tenofovir, were not detectable by RT-PCR in these tissues.[201]

The comparison in mRNA expression patterns of cervicovaginal and colorectal tissue transporters between human, animal models, and the comparison between human tissues and cell lines have implications on the utilization of the animal and cell models in PrEP testing and functionality study of cervicovaginal transporters. Non-human primates are considered as the most clinically relevant animal model to study STI transmission and prevention. The rabbit model has been recommended by FDA for topical (vaginal and rectal) PrEP product safety testing. The wild-type mouse model utilized in our study is also used in safety evaluations as a convenient *in vivo* model.[198,203,204] It has been shown that the toxicity profile of a number of

microbicide candidates in progesterone synchronized mice correlated well with clinical trial results.[199,200,205] In addition, the End1/E6E7, Ect1/E6E7, and VK2/E6E7 cell lines as well as the PM1 T cell line have been widely used in microbicide safety or efficacy evaluations. Since the drug efficacy and toxicity are dependent on drug level in the recipient tissues, the comparisons between human and models have provided useful information regarding the validity of those models especially when the test drug is a substrate of the highly expressed transporters. Additionally, the interspecies comparisons have provided clues for the selection of preclinical models for future functional characterization of cervicovaginal/colorectal tissue transporters. However, it should be noted that mRNA levels do not necessarily correlate with protein expression and activity. Functional studies will be necessary to confirm the observed interspecies differences in transporter mRNA levels between human and the preclinical models used in PrEP product evaluation. Mrp4 transporter mRNA level in rabbit cervix and vagina were found to be much higher than that in corresponding human tissues. However, rabbit vagina is lined with single-layer epithelium, which is different from the multi-layer epithelia of human vagina and ectocervix. This anatomical difference renders the permeability across rabbit vaginal epithelium higher than that of human vaginal and ectocervical tissues. Even if the Mrp4-mediated efflux activity in rabbit tissues is higher, the overall uptake and tissue exposure of Mrp4 substrates (e.g. tenofovir) may still be comparable between humans and rabbits. Therefore, current data on rabbit transporter mRNA levels is not against the FDA recommended use of rabbit model in the screening of topical PrEP products.

The conventional and real-time RT-PCR studies reported in this chapter were not without limitations, and strategies have been employed to address the shortcomings. Some primers used

in the PCR did not span the boundary of exons, rendering the risk of amplification from genomic DNA, and in turn may result in false positive results. To address this, DNase was utilized in the preparation of RNA samples for real-time RT-PCR, in order to remove the residual genomic DNA contamination. In addition, in some of the real-time RT-PCR analysis, the size of the PCR products was not confirmed by DNA electrophoresis. Although melt curve analysis following the amplification suggested the formation of single PCR product, it was possible that a non-specific amplification occurred instead of the target-specific amplification, so that the melt curve analysis still showed single amplification product during PCR. If this was the case, then positive detection of some transporters may just be false positive results. This limitation was resolved by examining the protein expression and/or transport function of the transporters with positive mRNA results. A third shortcoming of this study is associated with the difficulty in obtaining tissues from humans and non-human primates. A limited number of human cervicovaginal tissues was used in PCR experiments, and some experiments lack the most appropriate positive control tissues. For example, OAT1 and OAT 3 are highly expressed in kidney,[119] but human kidney could not be obtained for our study as positive control. The positive control used for all transporters in our study was human liver, but the OAT1 and OAT3 mRNA expression is known to be minimal in this tissue. Thus, using liver as positive control for these transporters could not eliminate the possibility that the OATs were expressed at high level in cervicovaginal and colorectal tissues but primers were inefficient to detect the expression. This concern has been addressed by similar findings reported by other groups using better control tissues. Nicol *et al.* also reported negative real-time RT-PCR detection of OAT1 and OAT3 in human endocervix, vagina, and colorectal tissue. In this study, human kidney was used as positive control, and high levels of OAT1 and OAT3 could be detected in the kidney.[206] The utilization of DNase, later

examination of protein expression of select transporters, and similar findings reported from other groups have enhanced the validity of our mRNA results described in this chapter.

Efflux transporters have become the focus of our study that aims to identify critical determinants of drug PK/PD, due to a number of reasons. First, efflux transporters displayed higher expression levels than many uptake transporters and drug metabolizing enzymes. The majority of CYP isoforms, such as CYP3A4, were not detectable or were expressed at much lower levels compared to the liver, as identified in our previous studies.[201] The relatively high mRNA levels of efflux transporters suggest that they may be more important regulators than uptake transporters or CYP enzymes in drug exposure in the cervicovaginal tissues. In addition to relatively high expression levels, extensive studies have tested the feasibility of inhibiting efflux transporters to increase drug exposure in tissues.[47,49,59,207,208] The mostly tested approach is co-administration of transporter inhibitors.[42, 43] Another promising approach is to encapsulate the drug into lipid nanoparticles so that the drug will enter the cell via alternative routes and the transporter efflux could be minimized.[208] There are a number of inhibitors and formulation strategies to utilize to modulate an efflux transporter in the scenario of PrEP. Many antiretroviral drugs are inhibitors of efflux transporters, such as protease inhibitors being potent P-gp inhibitors. In addition, there have been a lot of studies exploring nanoparticle formulations with diverse physicochemical properties for the incorporation of antiretroviral drugs. Compared to efflux transporters, there is much less information on the strategies available to enhance the uptake transporter function and get more drug molecules into the cell. Therefore, the relatively high expression levels of P-gp, BCRP, MRP4, 5, 7 in cervicovaginal tissues, the high relevance of

these transporters to antiretroviral drugs, and the availability of multiple approaches to overcome the efflux activity led us to focus on these efflux transporters in follow-up studies.

Based on the mRNA expression profile of the transporters examined, a number of antiretroviral drugs could potentially be affected by the positively expressed transporters. Based on the Biopharmaceutics Classification System (BCS) discussed in Chapter 1, three classes (Class 2, 3, 4) of antiretroviral drugs could be potentially affected by efflux transporters positively detected in cervicovaginal and colorectal tissues. Among the drugs from these three classes, nevirapine (MRP7), atazanavir (P-gp), ritonavir (P-gp), amprenavir (P-gp), lopinavir (P-gp), saquinavir (P-gp), darunavir (P-gp), indinavir (P-gp), tipranavir (P-gp), nelfinavir (P-gp) and raltegravir (P-gp) are from BCS Class 2; maraviroc (P-gp), lamivudine (BCRP), tenofovir DF (P-gp), tenofovir (MRP4, MRP7), didanosine (BCRP) are from BCS Class 3. Abacavir (P-gp, BCRP, MRP4), stavudine (BCRP, MRP5) and zidovudine (BCRP, MRP4) are substrates of transporters positively expressed in cervicovaginal and colorectal tissues, but their PK is not likely to be significantly affected because they belong to BCS Class 1. Etravirine belong to BCS Class 4, but the role of transporters in its PK is not clear because no transporter has been identified to transport this drug. Antiretroviral drugs from two classes (Class 3 and 4) are potentially affected by uptake transporters. In this dissertation, only ENT1 out of the tested uptake transporters showed cervicovaginal and colorectal expression level higher than liver. Among the drugs from these two classes, the only drug that can be transported by ENT1 is didanosine, which belongs to BCS Class 3. To summarize, based on the transporter expression profile revealed in by this study, 16 approved antiretroviral drugs are potentially affected by efflux transporters positively

expressed in cervicovaginal/colorectal tissues including P-gp, BCRP, MRP4, and MRP7; only 1 drug may be affected by the positively expressed uptake transporter ENT1.

In previous PrEP clinical and non-human primate studies, several factors have been suggested to increase the vulnerability to HIV sexual transmission and decrease the effectiveness of antiretroviral-based PrEP drug products. Two major factors were found to be hormones/hormonal contraceptive, and reproductive tract inflammation.[209-212] Macaques with progesterone implants displayed increased infection rate than non-administered macaques.[211] Contraceptives have shown varying effects on HIV infection.[212] Depo-Provera is the most widely used contraceptive in sub-Saharan Africa where the rate of HIV sexual transmission remains highest. African women who used Depo-Provera had significantly elevated HIV acquisition rate compared to those who used another contraceptive norethisterone enanthate (NET-EN).[212] In addition to hormones and contraceptives, human participants with bacterial vaginosis demonstrated increased rate of HIV sexual transmission compared to those with healthy reproductive tract.[209,210] The mechanism underlying the increased infection has been suggested to involve increased amount of submucosal immune cells and increased HIV receptor abundance on immune cell surface, when Depo-Provera and proinflammatory cytokines were present.[213] However, the effect of Depo-Provera and proinflammatory cytokines on tissue drug levels have not been examined. If Depo-Provera and BV-associated cytokines could alter transporter levels and decrease drug exposure in tissues and cells relevant to HIV transmission, then the effectiveness of PrEP drug products may be worsened in the participants who use Depo-Provera or have genital tract bacterial vaginosis.

It has been reported that hormones and proinflammatory cytokines could alter the expression and activity of drug transporters,[214-217] therefore the effect of these factors on transporter expression in transmission-related tissues and cells is worth exploring. Ideally, to study the effect of contraceptives, tissues should be collected from human or macaque subjects with and without contraceptive use; to test the effect of reproductive tissue inflammation, tissues should be collected from humans with and without ongoing inflammation such as bacterial vaginosis. However, due to the difficulty of collecting human and non-human primate tissues, only limited number of tissues were collected from humans and macaques, without knowing the information of menstrual cycle, hormone/contraceptive use, and inflammation status. Therefore, the effect of menstrual cycle and exogenous hormones/contraceptives/inflammation was tested in cell lines and mice. To test the effect of menstrual cycle, tissues were collected from mice at different stages of natural estrous cycle. To test the effect of hormones and contraceptives, cell lines were treated with MPA (active ingredient of Depo-Provera) and progesterone, and mice were treated with PMSG and Depo-Provera. To test the effect of inflammation, cell lines were treated with IL1β and IL8 solution, since these two cytokines were found to be significantly elevated in women with bacterial vaginosis, and presumably contribute to the inflammation status.[185-187]

In the mouse model, menstrual (estrous) cycle, PMSG, and Depo-Provera demonstrated regulatory effects on the expression of several efflux transporters in mouse cervicovaginal tissues, and the most prominent effects were observed on Abcb1a/Abcb1b (P-gp), Abcg2 (Bcrp) and Abcc4 (Mrp4). The impacts of these factors were found to be dependent on the type of transporter and type of tissue. The general tendency was that the mRNA expression of these transporters was higher at diestrus stage compared to the level at estrus stage, during the natural

estrous cycle. In addition, PMSG, the estrogen-stimulating hormone, decreased transporter expression compared to the transporter levels during natural cycle. On the contrary, Depo-Provera synchronization increased transporter expression compared to the transporter levels during the natural cycle. If these effects could be observed in humans, the menstrual cycle and the use of estrogenic hormone drugs or Depo-Provera could potentially result in intra-individual and/or inter-individual variability in tissue drug exposure and PrEP effectiveness. For example, in mouse endocervix and ectocervix, Abcc4 (Mrp4) level was significantly elevated at diestrus stage during natural estrous cycle, and was markedly up-regulated by Depo-Provera treatment. If these changes at mRNA level correlate with transport activity, the tissue exposure of topically or systemically administered TFV may be reduced under the up-regulated conditions compared to the conditions with unchanged or down-regulated Abcc4 level.

It would be tempting to understand the mechanisms underlying the observed transporter regulations by menstrual (estrous) cycle, PMSG, and Depo-Provera. With an understanding of these mechanisms we may be able to predict other untested factors that can regulate transporters in the tissues of our interest through the same mechanisms. Currently there are no published reports describing the effect of Depo-Provera, or its active pharmaceutical ingredient medroxyprogesterone acetate (MPA), on the expression of MRP4 transporter. Our results represent the first demonstration that mouse Mrp4 mRNA expression can be regulated by Depo-Provera, in cervicovaginal tissues. The mechanism may involve the expression and activity of NRs in cervicovaginal tissues. MPA has been demonstrated to bind and activate multiple NRs.[218] While MPA does not bind significantly to the NRs for estrogen and mineralocorticoids (ER and MR), it is an agonist of progesterone receptor (PR), androgen receptor (AR), and glucocorticoid

receptor (GR).[218] The approximate EC_{50} values were 0.01, 1, and 10 nM, for PR, AR and GR, respectively.[219] AR activation has been reported to up-regulate MRP4 gene expression in normal human prostate cells, prostate cancer cell line, and tumor tissue collected from prostate cancer patients.[220] After the binding of androgen, AR translocates into the nucleus, and binds the androgen response elements in the promoter region of ABCC4 (MRP4) gene to regulate MRP4 expression in normal human prostate tissue and prostate cancer cells.[221-223] The MRP4 expression in prostate tumor tissue was found to be higher than that in normal prostate cells, due to increased androgen signaling.[223,224] The treatment with bicalutamide, an AR antagonist, reduced MRP4 expression in LnCAP prostate cancer cell line.[223] In addition, prostate cancer patients receiving androgen ablation therapy had lower level of MRP4 expression in the tumor tissue, compared to the uncastrated prostate cancer patients.[223]

However, it should be noted that the effect of nuclear receptors is tissue-dependent.[225,226] The effect of the AR agonist (androgen) on MRP4 expression observed in prostate tissue and cell lines does not necessarily predict the effect of the AR agonist Depo-Provera in cervicovaginal tissues. In addition to AR, Depo-Provera binds to PR with high affinity *in vitro*.[219] However, currently there is no report demonstrating the role of PR in the regulation of MRP4 gene expression. In pregnant mice, liver Mrp4 mRNA expression was found to be elevated by 63% on gestational day 11, compared to the levels observed before pregnancy.[227] This implicated that progesterone and/or PR may be involved in MRP4 regulation, however more studies will be needed to confirm the involvement of PR. In addition to AR and PR, CAR, VDR, PPAR-α, Nrf2, and AhR have been demonstrated to regulate MRP4 expression,[110,228,229] but no interaction between these nuclear receptors and Depo-Provera have been reported. Based on this analysis,

more studies are warranted to confirm the role of AR and PR in the regulation of MRP4 in cervicovaginal tissues, and to investigate the involvement of other nuclear receptors in the Depo-Provera induced expression changes of MRP4.

In order to provide clues for the mechanism study of transporter regulation, and to evaluate the utility of different preclinical models in this kind of study, the mRNA expression of NRs was examined in the cervicovaginal and colorectal tissues of humans, macaques, and mice (Figures 2.17 to 2.19). Multiple NRs were found to express at moderate to high levels in these tissues across different species. These positively expressed NRs can respond to steroid hormones (e.g. ER-α), nutrients (e.g. RAR-α), and oxidative stress (e.g. Nrf2). The xenobiotic sensors, such as PXR and CAR, were absent in cervicovaginal tissues across different species (except low expression of PXR in macaque endocervix). However, PXR level in colorectal tissue was found to be high, and was comparable to its level in liver, in all species tested. This indicated that transporters in cervicovaginal and colorectal tissues may be regulated by different set of NRs, and may respond differently to different environmental factors. The expression pattern of NRs in cervicovaginal and colorectal tissues was generally comparable between humans, macaques, and mice. If the mRNA expression of the tested NRs correlates with their activity in the tissues examined in this study, then the comparable expression pattern between different species suggested that macaques and mice could be used as research models in the studies involving cervicovaginal and colorectal tissue NRs. The expression profile of NRs has provided clues for future investigation of transporter regulation mechanisms. For example, AR is a potential mediator of Depo-Provera regulation of Mrp4 transporter, as discussed above. In cervicovaginal tissues of human and Depo-Provera synchronized mice, AR level was close to or above 1/100 of

GAPDH in these tissues. In human and mouse colorectal tissues, AR level was more than 10 fold lower than its level in cervicovaginal tissues. Future studies could focus on the role of AR, in the Depo-Provera induced Mrp4 up-regulation. This could be achieved with AR agonists and antagonists, and possibly with AR knockout mice. In addition, the differential expression of AR between cervicovaginal and colorectal tissues may underlie the differential effects of Depo-Provera on Mrp4 expression between mouse cervicovaginal and colorectal tissues. Overall, the examination of NRs has provided useful information for the design of future mechanism studies and selection of animal models for these studies.

In addition to the mouse model, the effect of Depo-Provera was also tested on the two epithelial cell lines derived from human endocervix (End1/E6E7) and vagina (VK2/E6E7). Cervicovaginal cell lines responded differently to this treatment (Figure 2.15 and Figures 2.9-2.13). Depo-Provera did not alter the mRNA level of MRP4 in the three cell lines tested. However, it caused significant up-regulation of Mrp4 level in mouse cervicovaginal tissues. Several possibilities may explain the observed differences in the responsiveness to Depo-Provera. First, human and animal tissues are composed of multiple types of cells, and epithelial cells only constitute a small portion in the total amount of cells in cervicovaginal tissues. The observed effect in epithelial cell lines may not reflect the overall changes in the entire tissue. Another possibility is the difference in dose. The mice were administered with Depo-Provera (containing 3 mg of MPA) twice before tissue collection and expression analysis. The MPA concentration in the mice dosed with Depo-Provera remained unknown, but it was possible that MPA concentration available to mouse cervicovaginal tissues was different from the concentrations used to treat human epithelial cell lines (1 μM). The third possibility is that the regulation mechanism that Depo-Provera

activated in mouse tissues did not exist in human cervicovaginal epithelial cell lines. One possible mechanism that mediated the effect of Depo-Provera is nuclear receptor (NR) activation (discussed above). The interspecies difference in NR-mediated transporter regulation has been reported. Therefore, future studies will be needed to test the effect of Depo-Provera at different dosing levels, in more clinically relevant models, such as in the pigtailed macaques. Since many participants of PrEP clinical trials take Depo-Provera, the effect of this contraceptive on the cervicovaginal tissue transporters could be delineated by comparing the transporter expression and substrate drug PK data in Depo-Provera users with the data generated from other participants who do not take this contraceptive. If resources permit, this kind of clinical sample and data analysis will overcome the shortcomings of preclinical models, and generate the most clinically relevant information on Depo-Provera's effect, and will be valuable for decision making as to whether to adjust the PrEP drug dose in Depo-Provera users.

In addition to testing the effect of contraceptives/cytokines on transporter expression in cervicovaginal tissues, it is also important to comparatively examine the effect of these factors on transporter expression in HIV target immune cells. Since many antiretroviral drugs (nucleoside and non-nucleoside reverse transcriptase inhibitors, protease inhibitors, integrase inhibitors) act on intracellular targets within tissue-associated immune cells, the intracellular concentration of these drugs is critical for efficacy. In this chapter, a T cell line (PM1) exhibited differential response to contraceptive ingredients (MPA, P4) and proinflammatory cytokines (IL1β, IL8), compared to the epithelial cell lines End1/E6E7 and VK2/E6E7. For example, MPA has no significant effect on BCRP expression in End1/E6E7 and VK2/E6E7 cells, but caused significant decrease in BCRP level in PM1 cells. It is possible that the observed differences

between epithelial cell lines and PM1 was due to differential patterns of transporter regulation, between these cell lines. Nuclear receptors are potential mediators of the effects of contraceptives (discussed above), and human immune cells appeared to have different expression profile for a number of NRs tested in this study.[230] For example, PXR and CAR were undetectable in human cervicovaginal tissues (Figure 2.17), but it was found to positively express in CD4+ and CD8+ human T cells.[230] Future studies will need to test whether the differential changes in transporter expression is related to differential regulation of substrate drug exposure in different types of cells, and whether the changes in transporter levels could lead to altered drug efficacy in immune cells.

It should be noted that all the possible roles of cervicovaginal and colorectal tissue transporters are based on the assumption that positive mRNA levels correlate with efflux activity and significant role in drug pharmacokinetics. The mRNA expression of transporters does not necessarily correlate with their activity in microbicide absorption and disposition, and further work is needed to confirm the functional activity of the transporters that are moderately or highly expressed. Considering the number of transporters that showed high expression in cervicovaginal tissue, the information of protein localization, and mRNA/protein regulation will facilitate the prioritization and experimental design of the functional studies of cervicovaginal tissue/colorectal tissue/immune cell transporters. The information of the localization will help predict the specific role of transporters in microbicide pharmacokinetics.

The transporters localized in the epithelium may be able to limit the lumen-to-tissue drug distribution and tissue-to-lumen drug efflux, and therefore may be relevant to the

pharmacokinetics of drugs administered via both vaginal and systemic routes. The transporters localized within the venous endothelium such as BCRP may exert unidirectional control on blood-to-tissue drug distribution while not affecting lumen-to-tissue drug penetration. In addition, the localization information will help rationally select the model to study the functionality of transporters.

As mentioned above, a transporter could be located in different kinds of cells, such as epithelial cells and venous endothelial cells. If a transporter is exclusively located in one type of cell, e.g. endothelial cell, then the impact of transporters modulation on microbicide pharmacokinetics could be studied using primary or immortalized cell culture that reconstitutes the specific cell type *in vitro*. However, if a transporter is located in multiple cell types, then a clinically relevant animal model should be used to provide comprehensive understanding of the *in vivo* effect of transporter modulation on the cervicovaginal tissue exposure of substrates. This *in vivo* model must possess intact cervicovaginal tissues that are anatomically and physiologically comparable to the human female genital tract. Besides the protein localization, the understanding of the factors that can regulate the expression of transporters and activity in cervicovaginal tissues will also aid in the functional study. As reported in other tissues, the transporters which are highly expressed in vagina and cervix are subject to complex regulation mechanisms. One example is the effect of sex hormone on the expression and activity of transporters[217] Since sex hormones are deposited in the lower genital tract and their concentrations can be affected by various factors (race, age, menstrual stage, contraception choice),[231] it is possible that the expression and activity of transporters will change in different scenarios, causing inter- and intra-individual variability in cervicovaginal drug concentration and efficacy. It is therefore prudent to identify the scenarios

that transporters have differential expression/activity compared to the basal status, and understand whether this will result in a difference in the pharmacokinetic profile and efficacy of administered drugs that target the lower genital tract.

In conclusion, the studies in this chapter have provided a systematic evaluation of the expression profile of transporters in the cervicovaginal and colorectal tissues of human and PrEP animal models, as well as in the cell lines derived from human cervicovaginal tissues and immune system. In addition, this study has examined the effects of pathophysiological factors (PrEP relevant factors), including menstrual/estrous cycle, exogenous hormones/contraceptives/inflammatory cytokines, on the mRNA levels of transporters in transmission-related tissues and immune cells. The information generated from this study will facilitate the understanding of the role of transporters in PrEP drug pharmacokinetics, and will likely contribute to the development of novel strategies aimed for achieving adequate tissue levels of PrEP drug products. This study also provides critical information regarding rational experimental design and data interpretation, for animal and cell line models utilized in PrEP drug screening and future *in vivo* functional study of cervicovaginal/colorectal transporters. Nevertheless, this study is not without limitations. The mRNA level does not necessarily correlate with protein level and transport/enzyme activity. Future studies are warranted to examine the protein localization and regulation of the most important transporters in tissues, and to examine the functionality of the highly expressed transporters in antiretroviral drug PK/PD.

3 PROTEIN LOCALIZATION OF SELECT TRANSPORTERS IN CERVICOVAGINAL AND COLORECTAL TISSUES OF HUMAN AND ANIMAL MODELS

3.1 Introduction

In Chapter 2, the mRNA expression of drug transporters has been examined in tissues and cells relevant to HIV-1 sexual transmission. However, the mRNA levels do not necessarily correlate with transporter activity, and further work is needed to confirm the functional activity of the transporters and enzymes that are moderately or highly expressed.

The information on transporter protein localization will help rationalize the experimental design in the studies of transporter function. Transporters located in the epithelium presumably limit the lumen-to-tissue drug distribution and tissue-to-lumen drug efflux, and therefore may be a relevant factor in the pharmacokinetic profile of drugs administered via both vaginal and systemic routes. Transporters localized within the venous endothelium may affect the tissue-to-blood drainage of the substrate drugs already distributed into tissue. Given this potential impact on pharmacokinetics (PK), examination of transporter localizations is required information for appropriate experimental design and accurate interpretation of pharmacokinetic data obtained from different physiologic compartments.

Female users of the PrEP products have varying levels of sex steroid hormones (e.g. estrogens and progesterone) during their menstrual cycle. In addition, they frequently use contraceptives to avoid unwanted pregnancy. The information of transporter protein localization, under the influence of menstrual cycle, sex steroid hormones and hormonal contraceptives will enhance our understanding on the dynamics of transporter expression in PrEP participants. In addition,

the examination of the effect of these factors on transporter protein abundance and localization will help identify the scenario in which a transporter has the highest expression level and would most likely play a role in drug pharmacokinetics. This information will in turn guide the experimental design and prioritization of transporter function studies. As reported in other tissues, the transporters which are highly expressed in the vagina and cervix are subject to complex regulation mechanisms. One example is the effect of sex steroid hormones on the expression and activity of transporters. Since sex steroid hormones are deposited in the lower genital tract and their concentrations can be affected by various factors (race, age, menstrual stage, contraception choice), it is possible that the transporter expression and activity will change in different scenarios, causing inter- and intra-individual variability in cervicovaginal drug concentration and efficacy. It is therefore prudent to identify the scenarios that transporters have differential expression/activity compared to the basal status, and understand whether transporter modulation under this condition could result in improved tissue exposure of substrate drugs.

The studies described in this chapter aimed to examine the protein localization of drug transporters in the tissues associated with or relevant to HIV-1 sexual transmission, in human and animal models utilized in PrEP testing. P-gp, BCRP, and MRP4 were found to be moderately to highly expressed in cervicovaginal and colorectal tissues of human, macaque, and mice. Among other positively detected transporters, these three efflux transporters potentially affect the pharmacokinetics/pharmacodynamics (PK/PD) of largest number of antiretroviral drugs, as discussed in Chapter 1 and 2, and should be listed as priorities in further investigations of cervicovaginal and colorectal tissue transporters. Therefore, in this chapter, the protein localization of these three efflux transporters was examined in the endocervix, ectocervix, vagina,

and colorectal tissue of human, pigtailed macaque, and Swiss Webster mice, using immunohistochemical (IHC) staining. To examine the effect of menopause, the protein localizations of P-gp, BCRP and MRP4 were compared between human premenopausal and postmenopausal ectocervix. In order to evaluate the effect of menstrual cycle, exogenous hormones and contraceptives, the protein localizations of P-gp, Bcrp and Mrp4 were examined in mouse tissues collected at different estrous cycle stages and after the treatment of PMSG/Depo-Provera. The information revealed from this chapter confirms the mRNA results described in Chapter 2 at the protein level, and provides critical information on the experimental design and model selection of transporter function studies in next chapter.

3.2 Materials and Methods

3.2.1 Collection of human and animal tissues

The procurement of human, macaque, and mouse tissues were described in Chapter 2. Tissues were fixed in 10% neutral buffered formalin (10% NBF) for no less than 24 hours. The rabbit tissues were not stained due to the lack of appropriate primary antibodies for rabbit transporter detection.

3.2.2 Immunohistochemical staining

Immunohistochemical staining was conducted by the Research Histology Service of the University of Pittsburgh. Human, macaque, and mouse tissues (endocervix, ectocervix, vagina, and sigmoid colon/colorectum) were fixed in 10% neutral-buffered formalin for over 24 hours and embedded in paraffin. Five µm sections were made and de-paraffinized using xylene. Antigen retrieval was performed by steaming the slides in the pH9 retrieval buffer (Dako) for 40

minutes. The slides were treated with 3% H_2O_2 for 8 minutes, followed by blocking in Avidin/Biotin block solution (Vector) for 15 minutes and blocking in non-serum protein block for 10 min.

Primary antibodies (Table 3.1) purchased from Santa Cruz Biotechnology Inc. were applied to slides with overnight incubation at 4 °C. After washing with phosphate buffered saline solution containing Tween 20 (PBST), biotinylated secondary antibodies (Table 3.2) purchased from Vector Inc. were applied to the slides and incubated at room temperature for 30 min.

Table 3.1 Information of primary and secondary antibodies.

Transporter	**Primary antibody**		**Biotinylated secondary antibody (1: 200 IgG)**	
	Human and macaque samples	Mouse samples	Human and macaque samples	Mouse samples
P-gp	H-241 (1:15)	H-241 (1:15)	goat anti-rabbit	goat anti-rabbit
BCRP	BXP-21 (1:20)	BXP-53 (1:25)	horse anti-mouse	Goat anti-rat
MRP4	F6 (1:50)	M4I-80 (1:15)	horse anti-mouse	Rabbit anti-Rat

After the incubation with secondary antibodies, the slides were washed with PBST. ABC Elite reagents (Vector) were applied afterwards and slides were incubated for 30 minutes, followed by AEC chromogen (Skytec) incubation for color development. The slides were then counterstained with Hematoxylin and mounted with Crystal Mount (Sigma). In the negative control staining for a transporter protein in a given tissue, the non-immunized IgG purified from the species in which the primary antibody was raised was used instead of the primary antibody.

3.3 Results

3.3.1 Protein localization of P-gp in human and macaque cervicovaginal tissues

For P-gp staining in human tissues, the sigmoid colon was stained as a positive control tissue, since abundant P-gp protein expression has been reported for this tissue.[139] The apical membrane of the columnar epithelial cells of sigmoid colon was stained strongly positive, while the colonic vascular endothelial cells were not stained (Figure 3.1). This is in line with published studies and validated the IHC staining procedure in our experiments.

As discussed above, since multiple cell types may control the drug absorption and disposition in a given tissue, the columnar epithelial cells, stratified squamous epithelial cells, as well as vascular endothelial cells along the cervicovaginal tract were examined for their staining of transporter proteins. In endocervix, ectocervix and vagina, the epithelial cells facing the cervicovaginal lumen as well as the endothelial cells of the blood and lymphatic vessels running through the stromal tissue were stained positive (Figure 3.1). Among the epithelial cells of the three kinds of genital tract tissues, the stratified squamous epithelial cells in ectocervical tissue showed most intensive staining. In ectocervix, the basal layer of the ectocervical epithelium, which is composed of single layer of columnar cells, showed even more intense staining compared to the uppermost layers. Notably, the uppermost layers predominantly displayed plasma membrane staining for P-gp, while the basal layer of the epithelium displayed cytosol staining. This difference was possibly due to more abundant P-gp distribution in subcellular organelles in the basal layer epithelial cells, as reported in other cell types.[232,233]

The staining of the endocervical and vaginal epithelial cells appeared to be weaker than that of ectocervical epithelial cells (Figure 3.1). In addition, the difference in staining signal intensity between the uppermost and basal epithelial layers in human vagina was less evident than the difference observed in ectocervix. However, the staining of the stromal vascular endothelial cells in human endocervix and vagina was as strong as it was in ectocervix. The positive staining of P-gp in human lower genital tract and colorectal tissue corresponded to their moderate to high level mRNA expression revealed in Chapter 2.

In macaque tissues, the P-gp staining patterns were generally similar to those of human tissues (Figure 3.1). However, differences had been observed between the two species. In the epithelial cells of macaque ectocervix and vagina, the staining signal was found in the cytoplasm rather than preferentially distributed on the plasma membrane. Moreover, there was no clear difference in signal intensity between different layers of the epithelium, in macaque ectocervix and vagina. The similarity between human and macaque in P-gp staining was consistent with the similarity in mRNA expression between these two species. The staining results for human and macaque P-gp were summarized in Table 3.2.

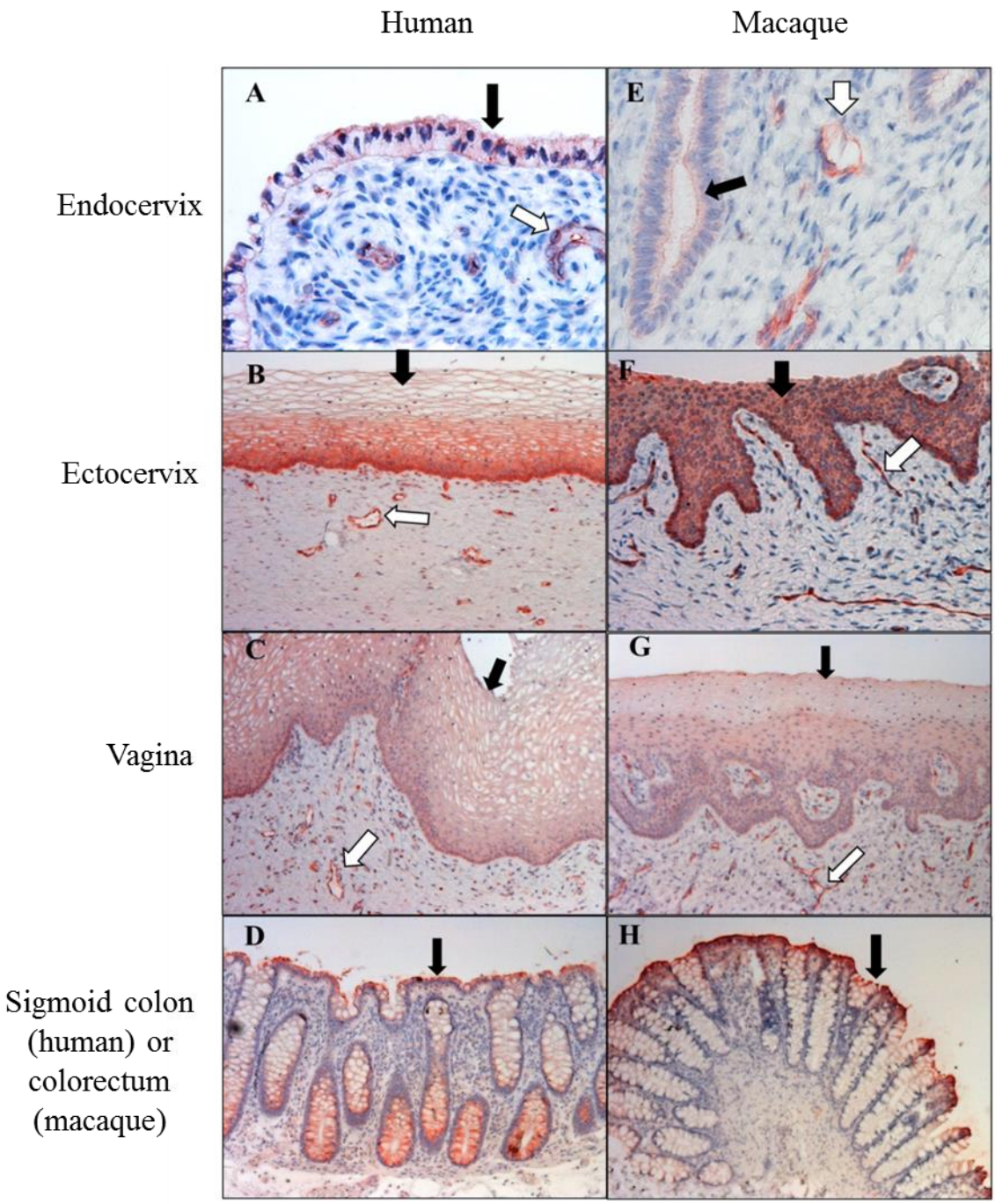


Figure 3.1 Localization of P-gp protein in human and macaque cervicovaginal tissues. Human and macaque tissues were fixed in 10% neutral buffered formalin (NBF) for no less than 24 hours, and embedded, processed, and stained for P-gp as described in Materials and Methods of this chapter. For endocervix, ectocervix and vagina, a representative picture from at least 3 tissue samples was shown above; for colorectal tissue, the staining of one tissue sample was shown above. Color development was with AEC, and red color indicates positive detection. A-D:

endocervix, ectocervix, vagina and sigmoid colon of women. E-H: endocervix, ectocervix, vagina and colorectum of pigtailed macaques. Black arrows: epithelial cells; white arrows: vascular endothelial cells. Magnification, 40 × for A and E, 10 × for B, C, D, F, G, H.

3.3.2 Protein localization of BCRP in human and macaque cervicovaginal tissues

For BCRP staining in human tissues, the epithelial cells of human sigmoid colon exhibited strong positive signal, while the staining of vascular endothelial cells was not observed in the colon (Figure 3.2). For the genital tract tissues, the staining was not observed in the plasma membrane of epithelial cells. However, positive staining signal was found in the nucleus of a subset of ectocervical and vaginal epithelial cells.

Intense staining was observed for the vascular endothelial cells within the stromal tissue of endocervix, ectocervix and vagina (Figure 3.2). Notably, strongly positive staining of blood vessels running through the epithelial layers of human ectocervix and vagina could be observed, which clearly distinguished these vessels from surrounding epithelial cells.

In macaque tissues, the BCRP staining patterns were generally similar to those of human tissues (Figure 3.2). However, differences were observed between the two species. The nucleus staining was not observed in the epithelial cells of macaque ectocervix and vagina. In addition, the epithelial cells of macaque colorectum appeared to have weaker staining compared to their human counterparts, while the staining of vascular endothelial cells was more obvious compared to those cells in human colon. The strong staining signal of BCRP in human and macaque genital tract tissues was in line with the high mRNA level of BCRP in these tissues, as described in Chapter 2. The staining results for human and macaque BCRP were summarized in Table 3.2.

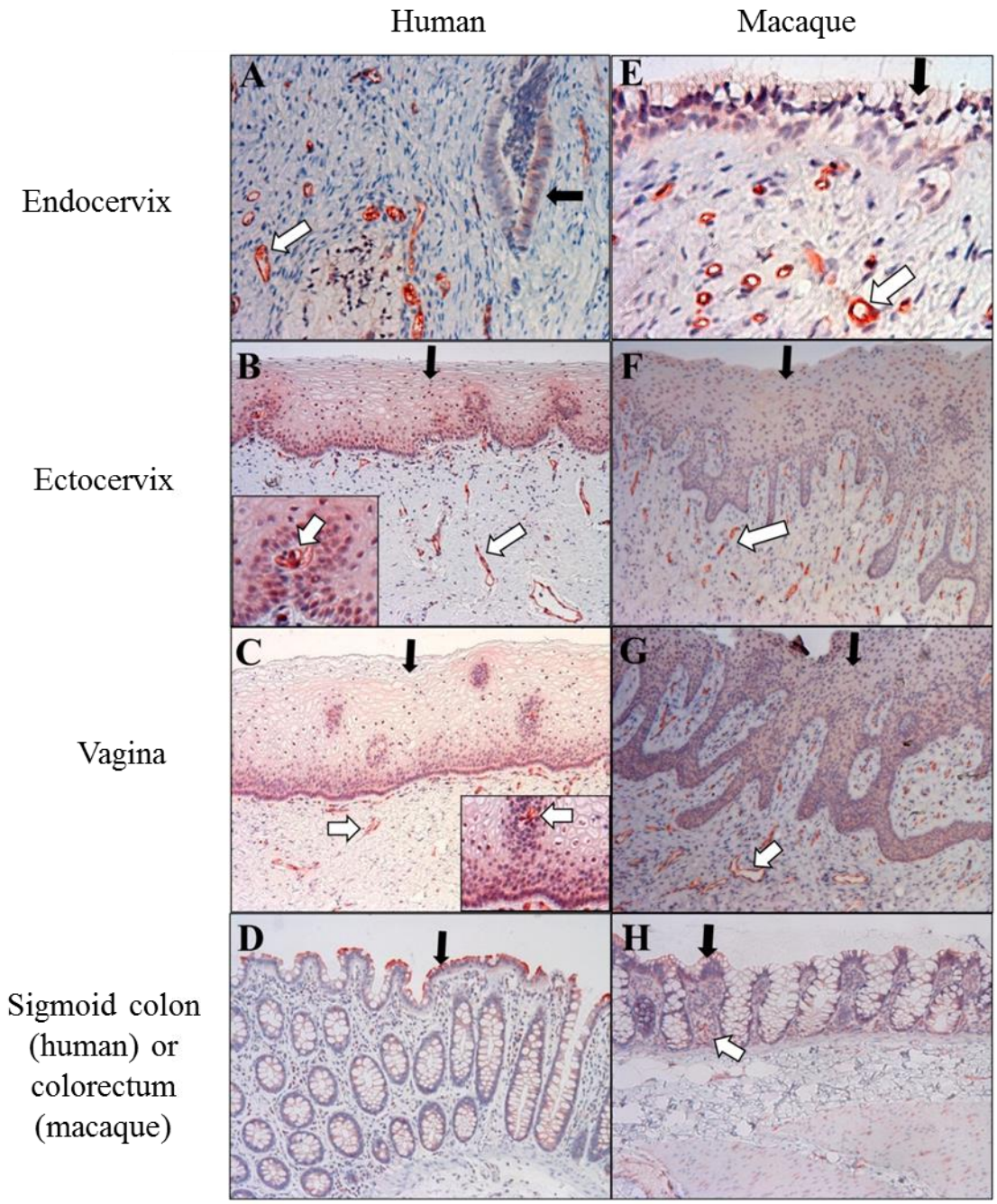


Figure 3.2 Localization of BCRP protein in human and macaque cervicovaginal tissues. Human and macaque tissues were fixed in 10% neutral buffered formalin (NBF) for no less than 24 hours, and embedded, processed, and stained for BCRP as described in Materials and Methods of this chapter. For endocervix, ectocervix and vagina, a representative picture from at least 3 tissue samples was shown above; for colorectal tissue, the staining of one tissue sample was shown above. Color development was with AEC, and red color indicates positive detection. A-D: endocervix, ectocervix, vagina and sigmoid colon of women. E-H: endocervix, ectocervix, vagina and colorectum of pigtailed macaques. The insets of B and C are enlarged epithelial areas

that contain intensely stained intraepithelial blood vessels and the positively stained epithelial nuclei. Black arrows: epithelial cells; white arrows: vascular endothelial cells. Magnification, 40 × for A and E, 10 × for B, C, D, F, G, H.)

3.3.3 Protein localization of MRP4 in human and macaque cervicovaginal tissues

For MRP4 staining in human tissues, the columnar epithelial cells of colorectum gave a moderately positive signal, while no staining was found in the vascular endothelial cells of the colon tissue (Figure 3.3).

Among genital tract tissues, the most intense staining was observed on the basolateral membrane of the columnar epithelial cells in endocervix. However, no staining signal can be readily observed in the vascular endothelial cells of endocervix (Figure 3.3). Compared to endocervix, the staining of ectocervical and vaginal epithelial cells appeared to be weaker. The staining of vascular endothelial cells in ectocervix and vagina appeared to be more obvious than that in endocervix (Figure 3.3).

In macaque tissues, the MRP4 staining of the columnar epithelial cells in macaque colorectum appeared to be less evident, while the vascular endothelial cells displayed stronger staining, compared to those cells in human sigmoid colon (Figure 3.3). In macaque endocervix, the staining pattern was identical to that of human endocervix. In macaque ectocervix and vagina, the cytoplasmic staining was the major staining pattern for MRP4, and no positive staining on the plasma membrane could be observed.

In human ectocervix, vagina and colorectum, the MRP4 staining appeared weaker than the signal of P-gp and BCRP, which was in line with the lower mRNA level of MRP4 in these tissues. The

stronger MRP4 staining signal in human endocervix and ectocervix compared to vagina was consistent with several fold higher MRP4 mRNA levels in endocervix and ectocervix, as demonstrated in Chapter 2. In addition, the similarity between human and macaque in MRP4 staining corresponded to the similarity in mRNA levels (Chapter 2). The staining results for human and macaque MRP4 were summarized in Table 3.2.

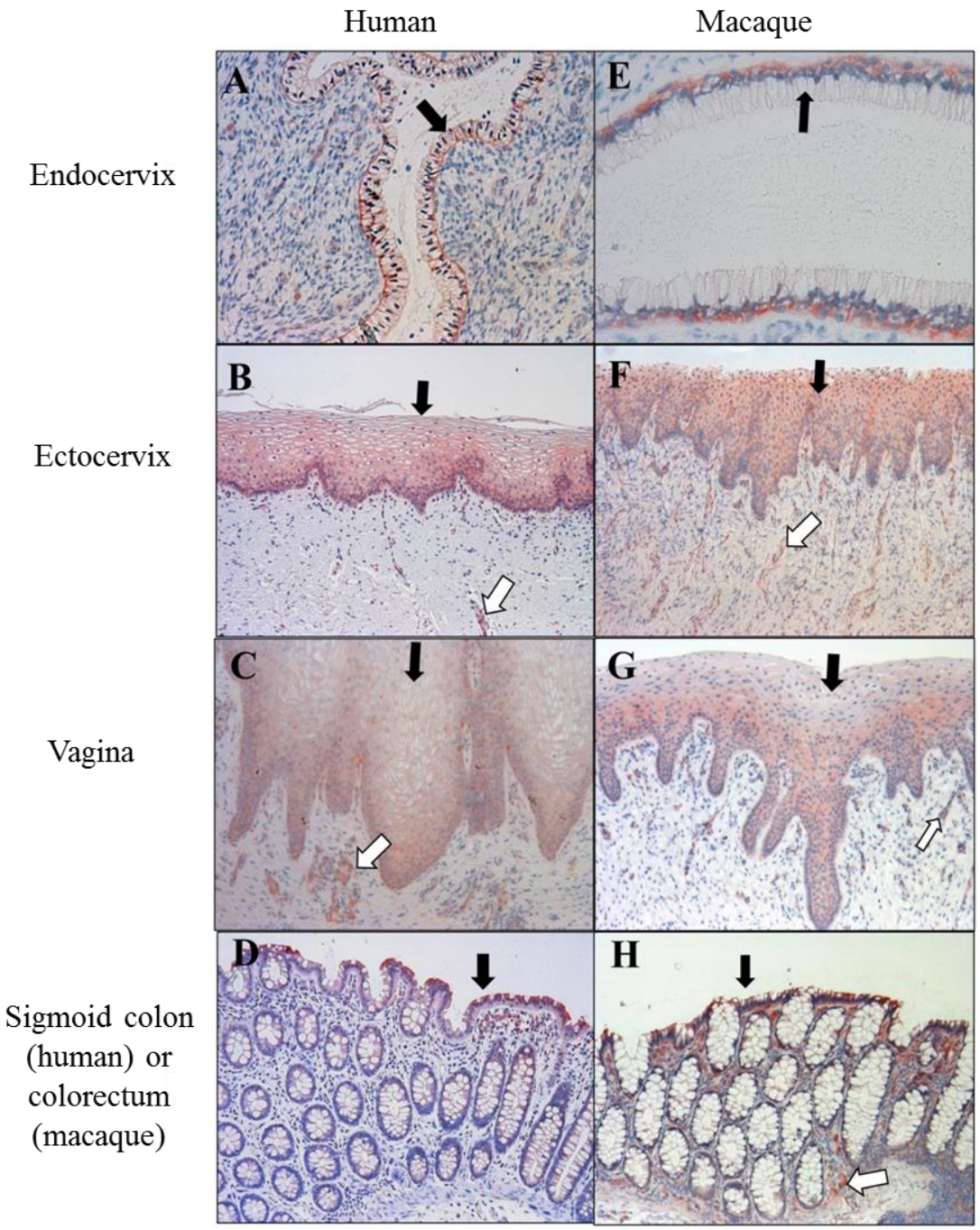


Figure 3.3 Localization of MRP4 protein in human and macaque cervicovaginal tissues. Human and macaque tissues were fixed in 10% neutral buffered formalin (NBF) for no less than 24 hours, and embedded, processed, and stained for MRP4 as described in Materials and Methods of this chapter. For endocervix, ectocervix and vagina, a representative picture from at least 3 tissue samples was shown above; for colorectal tissue, the staining of one tissue sample was shown above. Color development was with AEC, and red color indicates positive detection.

A-D: endocervix, ectocervix, vagina and sigmoid colon of women. E-H: endocervix, ectocervix, vagina and colorectum of pigtailed macaques. Black arrows: epithelial cells; white arrows: vascular endothelial cells. Magnification: 20 × for A, 40 × for E, 10 × for B, C, D, F, G, H.

Table 3.2 Summary of the immunohistochemical staining results of P-gp, MRP4 and BCRP in human and macaque tissues.

Tissue type	Cell type	*P-gp*		*MRP4*		*BCRP*	
		Human	*Macaque*	*Human*	*Macaque*	*Human*	*Macaque*
Endocervix	Columnar epithelial cells	●	●	●●	●●	○	○
	Vascular endothelial cells	●●	●●	○	○	●●	●●
Ectocervix	Squamous epithelial cells	●●	●	●	●	●	○
	Vascular endothelial cells	●●	●●	●	●	●●	●●
Vagina	Squamous epithelial cells	●	●	●	●	●	○
	Vascular endothelial cells	●●	●●	●	●	●●	●●
Sigmoid colon (human) or Colorectum (macaque)	Columnar epithelial cells	●●	●●	●	●	●●	●
	Vascular endothelial cells	○	○	○	●	○	●

○, not detected; ●, positively stained; ●●, strongly positive.

3.3.4 The effect of menopause on transporter protein localization in human ectocervix

To evaluate the effect of menopause on transporter protein abundance and localization in cervicovaginal tissues, IHC staining of P-gp, BCRP and MRP4 was performed on postmenopausal human ectocervix (Figure 3.4). Compared to the premenopausal tissues, the P-gp, BCRP and MRP4 protein expression appeared to be weaker and more diffuse in the epithelial layers. The condensation of transporter protein in the basal epithelial layers, which were seen for all the three transporters in premenopausal ectocervix, was not observed in postmenopausal tissues. However, the vasculatures remained densely stained for all the three transporters,

especially in the stromal part of tissues. Notably, there were more blood vessels running through the epithelium could be observed in postmenopausal ectocervix, compared to the premenopausal tissues.

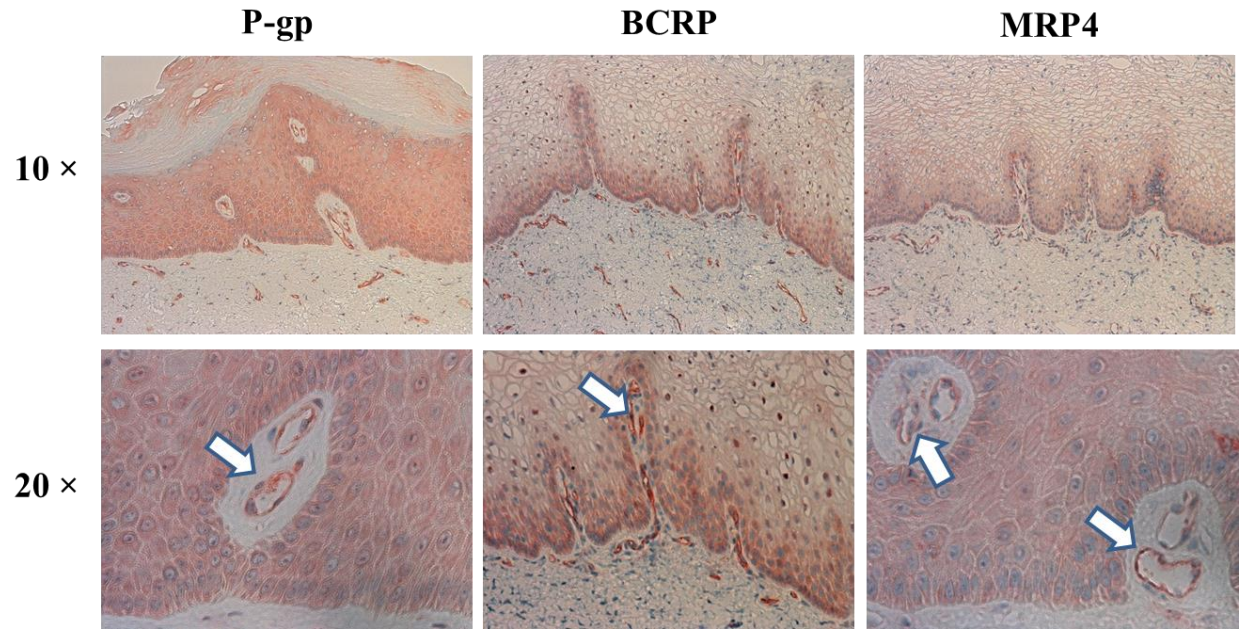


Figure 3.4 The effect of menopause on protein expression of P-gp, BCRP and MRP4 in human ectocervix.
Human postmenopausal ectocervical tissues were fixed in 10% neutral buffered formalin for no less than 24 hours, and embedded, processed, and stained for P-gp, BCRP and MRP4, as described in Materials and Methods of this chapter. For each transporter, 3 tissue samples (from 3 donors) were stained, and representative pictures were shown above. The staining of premenopausal ectocervix tissues for P-gp, BCRP, and MRP4 are shown in Figures 3.1, 3.2, and 3.3, respectively. Color development was with AEC, and red color indicates positive staining. Two magnifications were employed, 10 × for the first row to depict the overall staining pattern in both epithelium and stroma, and 20 × for the second row to focus on the staining of vascular wall of blood vessels running through the epithelial layers (white arrows).

3.3.5 Protein localization of P-gp in cervicovaginal tissues of naturally cycling and synchronized mice

During the natural estrous cycle, P-gp protein was distributed in both epithelium and stroma, in the three segments of female mouse genital tract (Figure 3.4). There was no marked difference in P-gp protein abundance and localization pattern. Compared to the natural estrous cycle, PMSG

treatment did not exert obvious effect on protein abundance or localization. Depo-Provera treatment did not affect P-gp protein in vagina. However, Depo-Provera increased the P-gp protein density in stroma and reduce P-gp density in epithelia of both endocervix and ectocervix (Figure 3.4).

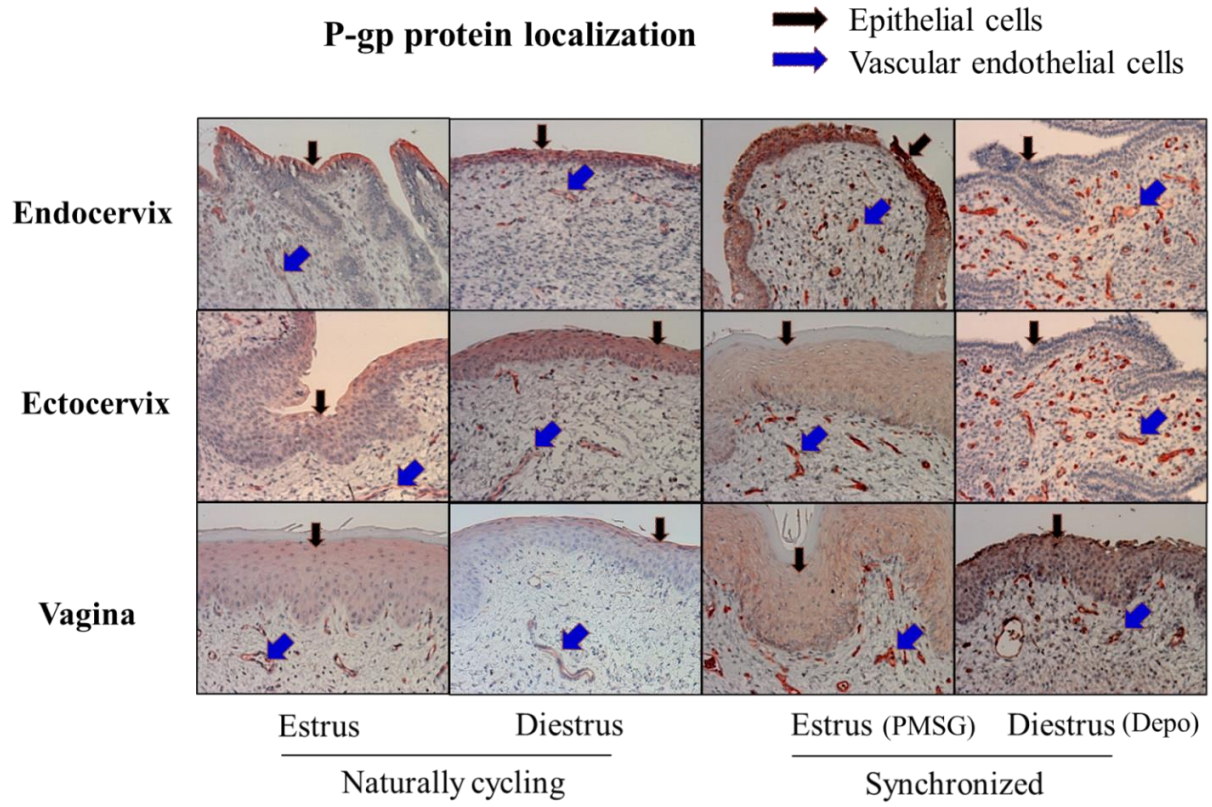


Figure 3.5 The effect of estrous cycle, PMSG and Depo-Provera on protein expression of P-gp (Abcb1a and Abcb1b) in mouse cervicovaginal tissues.
Tissues were collected from mice euthanized under the following 4 conditions: estrus and diestrus stages during the natural estrous cycle, synchronized estrus after PMSG treatment, synchronized diestrus after Depo-Provera treatment. Tissues were fixed in 10% neutral buffered formalin for no less than 24 hours, and embedded, processed, and stained for P-gp (antibody recognizes both mouse Abcb1a and Abcb1b), as described in Materials and Methods of this chapter. The majority of pictures shown are representative of the staining results from more than 3 mice. Color development was with AEC, and red color indicates positive staining. Black arrows indicate the position of epithelial cells, and blue arrows denote the positively stained vascular endothelial cells. Magnification, 20 × for all pictures.

3.3.6 Protein localization of Bcrp in cervicovaginal tissues of naturally cycling and synchronized mice

During the natural estrous cycle, Bcrp protein was primarily localized in the vessel wall of the stromal part of mouse endocervix and ectocervix (Figure 3.6). The epithelium of mouse vagina was also positively stained. There was no obvious difference in Bcrp protein abundance and localization between the two stages of natural estrous cycle, in all the three segments of mouse lower genital tract. Compared to the natural cycle, pregnant mare's serum gonadotropin (PMSG) synchronization did not seem to cause a significant change in endocervix and ectocervix, while Depo-Provera appeared to increase the protein density in these two tissues, especially in the stromal part. In addition, PMSG and Depo-Provera synchronization appeared to decrease Bcrp protein density in vaginal epithelium, compared to natural estrous stages.

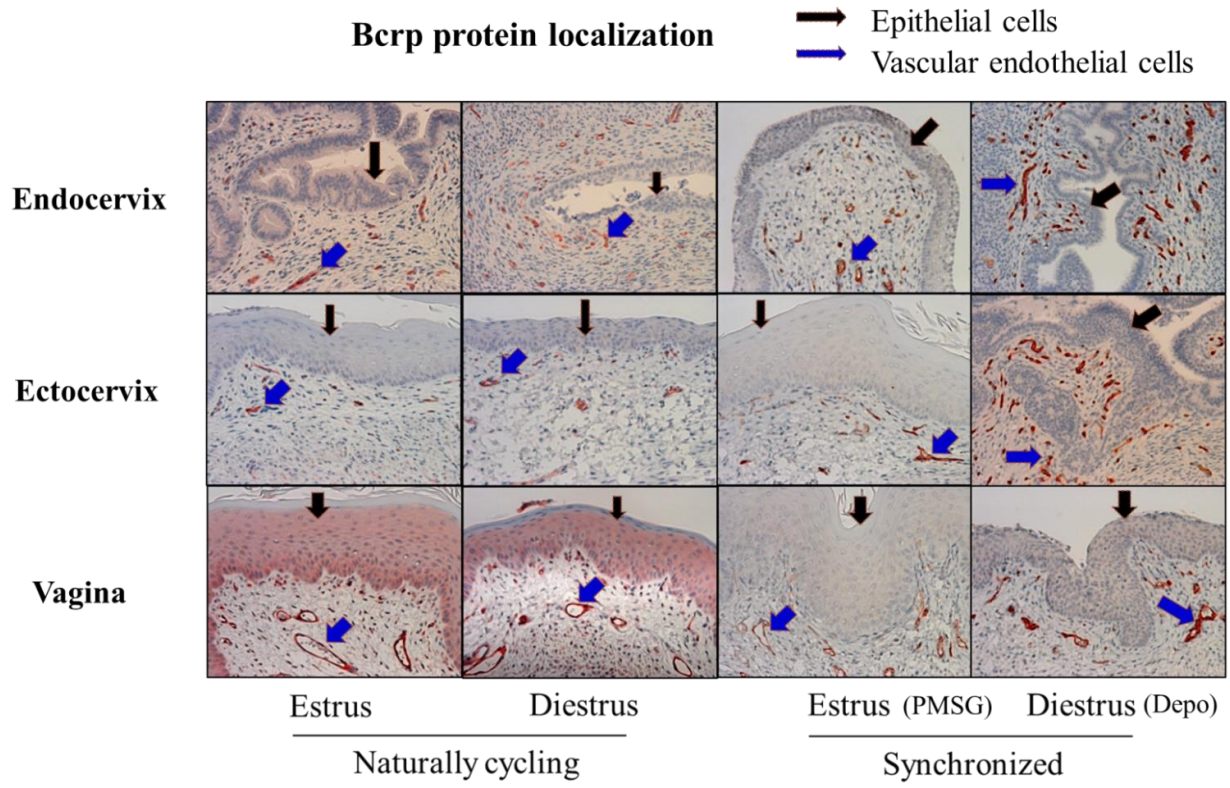


Figure 3.6 The effect of estrous cycle, PMSG and Depo-Provera on protein expression of Bcrp in mouse cervicovaginal tissues.
Mouse tissues were fixed in 10% neutral buffered formalin for no less than 24 hours, and embedded, processed, and stained for Bcrp, as described in Materials and Methods of this chapter. The majority of pictures shown are representative of the staining results from more than 3 mice. Color development was with AEC, and red color indicates positive staining. Black arrows indicate the position of epithelial cells, and blue arrows denote the positively stained vascular endothelial cells. Magnification, 20 × for all pictures.

3.3.7 Protein localization of Mrp4 in cervicovaginal tissues of naturally cycling and synchronized mice

During the natural estrous cycle, Mrp4 protein in mouse endocervix and ectocervix was primarily found at the diestrus stage, while the staining at estrus stage was not readily observed. Weak staining of Mrp4 protein was found in epithelium and stroma in mouse vagina at both estrus and diestrus stages. Compared to the natural estrous cycle, PMSG treatment decreased the epithelial and stromal abundance of Mrp4 protein in mouse endocervix, ectocervix and vagina.

Depo-Provera treatment markedly increased Mrp4 density in the epithelial layers, while decreased Mrp4 density in the stromal part, in all three tissue segments, compared to the natural estrous cycle.

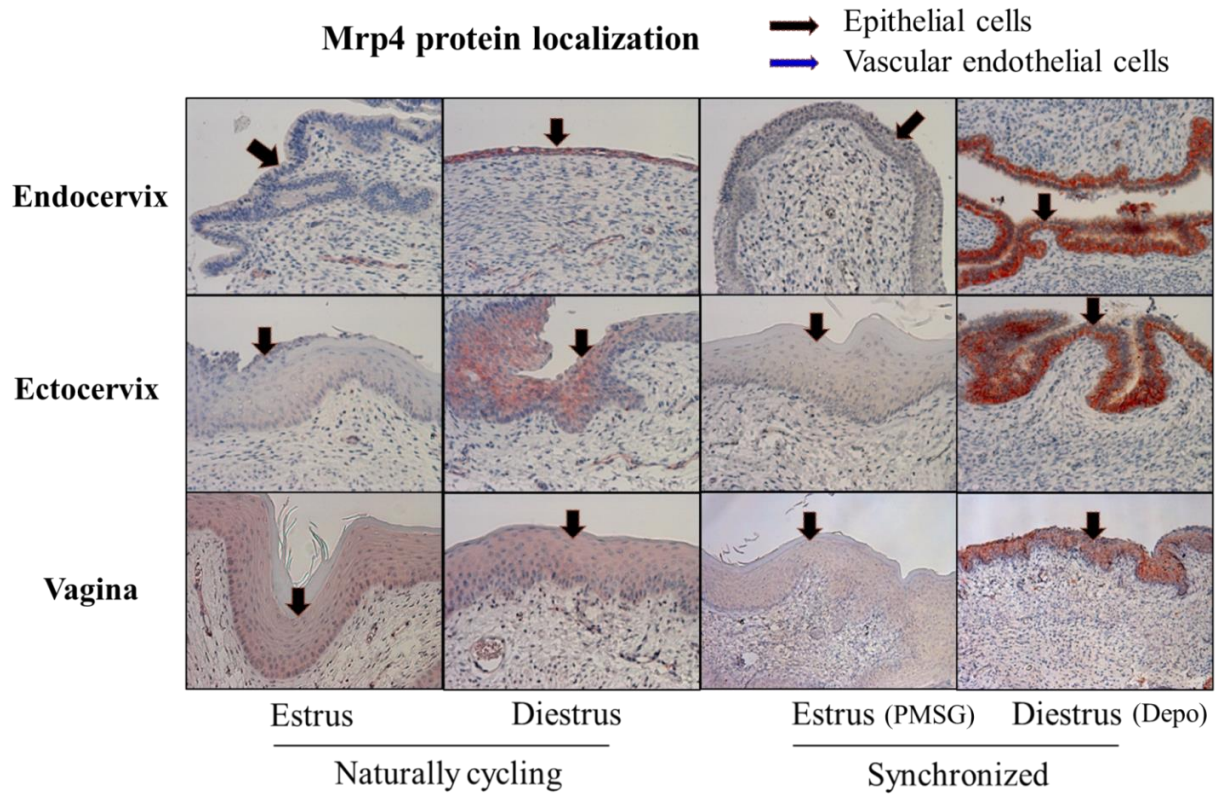


Figure 3.7 The effect of estrous cycle, PMSG and Depo-Provera on protein expression of Mrp4 in mouse cervicovaginal tissues.
Mouse tissues were fixed in 10% neutral buffered formalin for no less than 24 hours, and embedded, processed, and stained for Mrp4, as described in Materials and Methods of this chapter. The majority of pictures shown are representative of the staining results from more than 3 mice. Color development was with AEC, and red color indicates positive staining. Black arrows indicate the position of epithelial cells, and blue arrows denote the positively stained vascular endothelial cells. Magnification, 20 × for all pictures.

3.4 Discussion and Conclusion

The protein expression examined in this chapter confirmed the transporter mRNA results presented in Chapter 2. Although IHC staining is not a quantitative approach for the

measurement of protein level, the results here address the limitations of the RT-PCR approach adopted in Chapter 2.

The IHC staining results once again confirmed the positive mRNA expression of three most important efflux transporters, at protein level. P-gp, BCRP, and MRP4 were positively detected at mRNA level, in the cervicovaginal and colorectal tissues of humans, macaques, rabbits, and mice. The cervicovaginal levels were moderate or high compared to the levels in colorectal tissue and liver. In this chapter, the positive protein expression of these transporters in human, macaque, and mouse tissues was demonstrated using IHC staining. In addition, the protein abundance of P-gp, Bcrp, and Mrp4 in mouse cervicovaginal tissues generally correlated well with their mRNA levels under the influence of estrous cycle, PMSG, and Depo-Provera, as reported in Chapter 2.

The IHC staining of transporters presented provides valuable information on transporter localization, which cannot be revealed by RT-PCR. For example, the transporter mRNA levels did not significantly differ between pre- and postmenopausal human ectocervix (Chapter 2), however the differences at protein level has been found for P-gp, BCRP, and MRP4. Although the protein expression on stromal vessel walls remained unchanged for these three transporters (Figure 3.4), the protein expression appeared to be more diffuse in the epithelial layers in postmenopausal tissues. In addition, the IHC staining of mouse P-gp demonstrated that Depo-Provera treatment not only increased P-gp protein density in mouse endocervix and ectocervix, but also decreased the epithelial P-gp abundance in these two tissues (Figure 3.5).

However, it should be noted that positive mRNA and protein expression of cervicovaginal transporters do not necessarily predict their significant role in antiretroviral drug pharmacokinetics. In addition, the differential expression patterns of cervicovaginal transporters, under different conditions (menstrual cycle, exogenous hormones and hormonal contraceptives), do not necessarily result in differential levels of transporter activity. Post-translational modifications represent an additional mechanism of transporter regulation, sometimes resulting in the transport activity inconsistent with the protein expression level. Therefore, it is crucial to examine the function of the positively expressed transporters in cervicovaginal and colorectal tissue exposure of substrate drugs.

The region-specific characterization of transporter protein localization could inform the experimental design of studies on transporter function. The transporters examined in this study are localized in multiple cell types, including columnar (glandular) epithelial cells, squamous epithelial cells, and vascular endothelial cells. This suggests that each transporter may affect various aspects of substrate absorption and disposition. For topically (vaginally) administered drugs, the efflux transporters located on the luminal (columnar and squamous) epithelial cells provides a mechanism that can directly limit drug penetration into tissue, which could be similar to that observed for the enterocytes lining the small intestine.[49] As the columnar (glandular) epithelial cells are responsible for the secretion of mucosal fluid, the transporters located in this type of cell likely will affect the secretion of absorbed drugs from tissue to the mucosal fluid. The transporters located on the venous/lymphatic endothelium may have a role in the blood/lymphatic drainage from tissue to systemic blood/lymph circulation. For orally administered drugs, the transporters on the venous/lymphatic endothelium may limit the

distribution from blood/lymph to cervicovaginal tissues, and the transporters on glandular epithelial cells may function to efflux the drug from tissue to lumen. Therefore, when conducting studies to investigate transporter function in tissue drug distribution, its role in both topically (vaginally or rectally) and systemically administered drugs should be examined.

Drug absorption and disposition in the cervicovaginal and colorectal tissues is a complex process, which can be affected by a number of factors, including mucus secretion, systemic blood perfusion and drainage, as well as the regional blood flow between tissues.[234] Therefore, when studying a transporter's role in PrEP drug pharmacokinetics, it is critical to understand its comprehensive role in controlling drug exposure in the entire tissue and not just its function in a specific type of cell. When utilizing *in vitro* models to study transporter function, it is necessary to employ multiple cell lines corresponding to the multiple cell types that carry transporter protein in female genital tract. When utilizing animal models for functional characterization, it is prudent to ensure that the animal's genital tract is anatomically similar to human tissues, and patterns of transporter expression and localization are comparable to those patterns in human.

In this study, pigtailed macaques showed comparable expression and localization patterns of cervicovaginal P-gp, MRP4 and BCRP as compared to humans. Macaque is considered to be a biologically relevant model for PrEP efficacy assessment.[198,235,236] The similarity in transporter expression between humans and macaques also confirmed the utility of macaque model in microbicide efficacy and/or safety testing, especially when the three efflux transporters will affect the absorption and disposition of the tested drug.

Depo-Provera synchronized Swiss Webster mouse model has been used by researchers to evaluate the safety of vaginally administered PrEP products (microbicides).[199,200] Although the mRNA and protein expression of transporters in the Swiss Webster mice were not identical to those in human tissues, the mouse model is comparable to human in certain aspects. For example, the Depo-Provera synchronize mice showed strongly positive expression of Mrp4 in the epithelium of endocervix, which is similar to MRP4 expression pattern in human endocervix. In addition, the complexities of transporter-mediated drug distribution in tissues require an *in vivo* model for comprehensive understanding of transporter function, and the mouse model represents a convenient platform for proof-of-concept studies of transporter function *in vivo*. Future studies are warranted to examine the transporter expression in the cervicovaginal and colorectal tissues of humanized mice with transplanted human liver and lymphocytes,[237] thus both pharmacokinetics and efficacy studies can be conducted in the mouse model.

In conclusion, the studies presented in this chapter have characterized protein expression of three most relevant efflux transporters in the lower genital tract of humans, pigtailed macaques, and mice. The results have confirmed positive transporter expression in cervicovaginal tissues at protein level. In addition, these studies have confirmed the effect of menstrual (estrous) cycle, PMSG, and Depo-Provera on transporter protein abundance and localization, in cervicovaginal tissues. The comparison between human and animal/cell models in transporter protein expression provides critical information regarding the utility of these models to study cervicovaginal tissue transporter function. These results will inform the experimental design and data interpretation of future studies of transporter function.

4 FUNCTIONAL ROLE OF MRP4 TRANSPORTER IN THE DISTRIBUTION OF TENOFOVIR INTO MOUSE CERVICOVAGINAL AND COLORECTAL TISSUES

4.1 Introduction

Previous chapters have revealed the positive expression of several efflux transporters in the cervicovaginal and colorectal tissues of human and animal models, at mRNA and protein levels. The next required step is to investigate whether these transporters are functional in the tissue distribution of antiretroviral drugs. The information generated from such functional characterizations will enhance the understanding on critical determinants of drug exposure in those tissues relevant to HIV-1 sexual transmission, and facilitate PrEP optimization in multiple aspects.

Tenofovir (TFV) is an extensively investigated drug in both oral and topical PrEP.[10,238] It is a nucleotide reverse transcriptase and prevents HIV-1 infection by interfering with the reverse transcription of viral RNA in host immune cells. In oral PrEP, it has been tested in the prodrug form TFV disoproxil fumarate (TDF), in combination with emtricitabine. The combination regimen Truvada has been approved by the FDA for the prevention of HIV-1 sexual transmission between serodiscordant couples (one partner in the couple is HIV-1 positive while the other partner is negative). In the development of vaginal microbicides, it has been tested in dosage forms including gel, ring, and tablet. A 1% TFV gel showed effectiveness in reducing the HIV-1 acquisition rate by 39% in a Phase 2b trial.[13]

Strategies are being actively pursued to enhance the effectiveness of antiretroviral-based PrEP products for HIV-1 prevention,[12] and long-acting dosage forms are undergoing development. Although the utilization of long-acting products could avoid the low adherence issue observed in PrEP clinical trials,[12] challenges remain for the successful development of PrEP products. The effective *in vivo* drug concentration for HIV-1 prevention remains unknown,[239] and multiple barriers exist toward achieving sufficient drug exposure in the tissue-associated immune cells, even with topically applied microbicides.[33] Therefore, it is urgent to understand critical physiologic determinants of TFV exposure in cervicovaginal and colorectal tissues, to inform the development of novel strategies that can achieve maximally tolerated drug exposure and further enhance PrEP effectiveness. MRP4 and MRP7 efflux TFV, and MRP4 is the major efflux transporter.[84,113] OAT1 and OAT3 are major uptake transporters for TFV.[86,113] As demonstrated in Chapters 2 and 3, MRP4 and MRP7 were expressed at moderate to high levels, while OAT1 and OAT3 were not detectable, in human cervicovaginal and colorectal tissues. The IHC staining revealed that MRP4 protein was localized in the epithelial cells of human endocervix and colorectal tissue, and in the epithelial and vascular endothelial cells in human ectocervix and vagina. Based on these results, it is highly possible that MRP4 plays a role in TFV distribution into the cervicovaginal and colorectal tissues, and this should be examined in transporter function studies.

Although previous chapters have revealed the positive mRNA and protein expression of multiple transporters including MRP4, its *in vivo* role remains largely unknown. Functional studies are necessary to confirm the *in vivo* role of cervicovaginal and colorectal tissue transporters in controlling local tissue distribution of TFV. Even if the positively expressed MRP4 possesses

activity, it does not necessarily exert a significant impact on the pharmacokinetics of TFV in every tissue where positive expression was seen. TFV entry and retention in a given tissue is determined by multiple factors, including the passive transcellular and paracellular permeability of TFV, blood flow between tissues and systemic compartment, abundance and viscosity of cervicovaginal fluid, and binding to tissue proteins. Therefore, the study of the role of MRP4 in TFV distribution is warranted to confirm MRP4 function in the tissues with positive expression.

Based on the characterization of MRP4 mRNA and protein expression in the tissues of human and animal models, the Depo-Provera synchronized mouse was selected for the study of Mrp4 function in cervicovaginal and colorectal tissues. This model has been previously utilized by some researchers to evaluate the safety of vaginal microbicides, and has shown good correlation with the clinical results for several microbicide candidates.[199,200] The Mrp4 expression in mouse cervicovaginal and colorectal tissues were tested at two different stages of the natural estrous cycle, and under the synchronization by PMSG or Depo-Provera, which were described in Chapters 2 and 3. The mRNA and protein expression of cervicovaginal tissue Mrp4 was highest after Depo-Provera synchronization, compared to the mice undergoing natural cycling or synchronized by PMSG, suggesting the Mrp4 function may be most prominent under this condition. Efflux transporters, epithelial layer, intercellular tight junction proteins constitute the major component of the physiologic barrier against drug distribution from vaginal lumen to tissue and plasma. After Depo-Provera treatment, the cervicovaginal epithelium was evidently thinned, and it has been reported that tight junction protein abundance may be decreased after the treatment of progesterone-based agents. Under this condition, the role of Mrp4 transporter may become more evident in the overall permeation barrier function. In addition, the effect of co-

administration of Mrp4 inhibitor on substrate drug penetration may be most evident under this condition. This does not only apply to the vaginal administration, but may also be true in the systemic administration of substrate drugs, since the drug distribution from blood to cervicovaginal tissue and fluids also needs to go through the similar set of tissue barriers.

Although the expression level and protein localization of Mrp4 in mouse tissues were not identical to those in human tissues, the mouse model represents a convenient *in vivo* model for proof-of-concept studies of transporter function. In this chapter, the function of the Mrp4 transporter in the cervicovaginal tissue distribution of vaginally and systemically administered TFV is determined. In vaginal dosing studies, Depo-Provera synchronized mice were administered with vaginal gel containing TFV, with or without the Mrp4 inhibitor MK571. In systemic dosing studies, the mice were administered intraperitoneally with TFV solution, with or without MK571. The TFV concentrations in cervicovaginal lavage (CVL), cervicovaginal tissues and colorectum, as well as plasma were measured to delineate the effect of Mrp4 inhibition on TFV distribution in these three compartments. The results obtained from this study will provide direct evidence of Mrp4's role in TFV distribution into mucosal tissues relevant to HIV-1 sexual transmission.

4.2 Materials and Methods

4.2.1 Preparation of vaginal gels and drug/chemical solutions for mouse administration

^{3}H-TFV was purchased from Moravek Biochemicals and Radiochemicals Inc.. Two batches of ^{3}H-TFV stock were used, and the specific activity of the stock solution was either 13.3 Ci/mmol or 8.8 Ci/mmol. The radioactivity of the stock solution (EtOH:H_2O = 1:1) was 1 mCi/mL for

both batches. For vaginal gel administration, 20 µL of gel was administered intravaginally to each mouse, using a 1 mL disposable syringe (BD Biosciences) sequentially capped with a 200 µL pipette tip and a 10 µL tip, as shown in Figure 4.1 below.

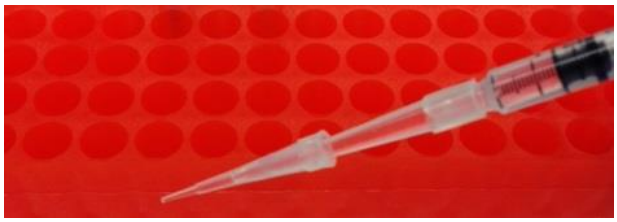


Figure 4.1 The syringe used for gel administration into mouse vagina.

For mouse vaginal administration, 2.7% universal placebo gel (pH4.4) was used as a base to mix with TFV and/or MK571. Two kinds of gel were formulated for the pharmacokinetic (PK) study, and three kinds of gel were formulated for the safety study. For each kind of gel listed in Table 4.1 below, at least 500 µL was prepared, and the amount of each ingredient was shown in the table. Gel administration in the mouse model is described in the next section (4.2.2) of this chapter.

Table 4.1 Preparation of gels for mouse PK and safety evaluations via the vaginal route

Ingredients	Amount added (µL)				
	PK study		Safety study		
	TFV gel	(TFV+MK571) gel	TFV gel	(TFV+MK571) gel	4% N-9 gel
^{3}H-TFV stock (8.8 Ci/mmol, 1 mCi/mL)	25	25	0	0	0
0.5% TFV solution (non-radiolabeled)	7.8	7.8	9.4	9.4	0
7.5 mg/mL MK571 saline solution	18	0	0	18	0
Saline (0.9% NaCl)	0	18	0	0	0
Nonoxynol-9 (N-9)	0	0	0	0	20
2.7% universal placebo gel	449	449	490	472	480
Total volume (µL)	499.8	499.8	499.4	499.4	500

Twenty µL of gel was administered to each mouse.

The components of the universal placebo gel used to formulate the gel products in mouse studies were shown in Table 4.2 below. Sorbic acid was added into water and mixed until fully dissolved. Sodium chloride was added, and pH was determined. If the pH was different from the target pH4.4, then 1M NaOH or HCl was added to adjust the pH. Hydroxyethyl cellulose was then slowly added to the solution with a mixer, and water is added to adjust the final total weight after all the ingredients were fully dissolved.

Table 4.2 Preparation of universal placebo gel

Ingredient	Amount added to make 100 g gel
Sorbic acid	0.1
Sodium chloride	0.85
Hydroxyethyl cellulose 250 HX	2.7
Sodium hydroxide 18%	As needed
MilliQ water	Supplement to 100
Total weight (g)	100

4.2.2 Mouse administration and sample collection

All animal procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC). Female Swiss Webster mice (6 weeks old, around 23 g body weight) synchronized with Depo-Provera were used for all experiments in this chapter. To synchronize the mice into diestrus stage, mice were subcutaneously (SC) injected twice with Depo-Provera (Pfizer Inc.), on Day 1 and Day 5, at the dose of 3 mg per mouse. Synchronized diestrus stage was reached on Day 8, and all the pharmacokinetic experiments were performed

on this day. The diestrus stage of mouse estrous cycle was confirmed when vaginal smears were found to contain predominantly leukocytes, as described in Chapter 2.

To study the functional role of Mrp4 transporter in the PK of vaginally administered TFV, synchronized mice were divided into 3 groups. The gels used in these experiments are listed in Table 4.1 above. Group 1 mice were dosed intraperitoneally (IP) with 100 µL of saline, followed by 20 µL of TFV gel dosed 30 min later vaginally; Group 2 mice were dosed IP with 100 µL of saline containing 7.5 mg/mL MK571, followed by 20 µL of TFV gel dosed 30 min later vaginally; Group 3 mice were dosed IP with 100 µL of saline, followed by 20 µL of (TFV+MK571) gel dosed 30 min later vaginally. The determination of TFV dosing level in mice was based on the measured TFV concentration in human cervicovaginal tissues several days post gel administration in the pharmacokinetic studies of 1% TFV gel.[21] The purpose of selecting this dose is to mimic the situation in which the tissue drug concentration is dramatically decreased from the initial level, and increasing drug exposure would most likely enhance microbicide effectiveness. The selection of MK571 dose was based on published animal studies with this inhibitor. In these studies, MK571 dose ranged from 1 to 100 mg/kg, and MK571 was administered through oral or intravenous routes.[240-242] In this chapter, 0.75 mg MK571 was administered IP to each mouse weighed around 23 g (32.6 mg/kg). This is an intermediate dose that was supposed to result in adequate levels in cervicovaginal and colorectal tissues for transporter inhibition, without causing obvious toxicity. Five minutes prior to vaginal gel dosing, mice were injected IP with 100 µL of saline containing 10 mg/mL ketamine HCl (Henry Schein Animal Health) and 20 mg/mL xylazine (Sigma-Aldrich), to sedate the mice and facilitate vaginal gel administration. The dosed mice were euthanized with CO_2 followed by cervical

dislocation, at 0.5 h and 1 h post gel administration, for all the three groups. The euthanized mice were immediately subject to the collection of cervicovaginal lavage (CVL). CVL was obtained through 8 sequential vaginal washes with 25 µL of saline (200 µL in total), using a 200 µL pipette tip with blunt-end. Around 0.25 cm of the sharp end of the tip was cut using a scissor to account for the high viscosity of the lavage. Blood was collected from inferior vena cava (IVC) and heart, using 1 mL disposable syringe capped with heparinized 25G needles. The collected blood was centrifuged at 5000 rpm for 5 min (Eppendorf MiniSpin Plus), and 100 µL of the supernatant was collected as plasma samples for further analysis. Mouse tissues including uterus, endocervix, ectocervix, vagina, colorectum, and kidney were collected using a surgical scissor. The harvested tissues were rinsed with saline, and the surface water was gently absorbed using gauzes. The tissues were then put into 1.5 mL tubes, and the weight of a tissue sample (mg) was obtained by subtracting the weight of the empty tube from the weight of the tube with the tissue.

To study the functional role of Mrp4 transporter in the PK of IP administered TFV, synchronized mice were divided into two groups. Group 1 mice were IP administered with 100 µL of saline, followed by IP injection of 100 µL of ^{3}H-TFV saline solution (0.94×10^{-5} mmol/mL, 125 µCi/mL) 15 min later. Group 2 mice were IP administered MK571-containing saline (7.5 mg/mL), followed by the same ^{3}H-TFV solution in Group 1. The determination of TFV dosing level in mice was based on the measured TFV concentration in human cervicovaginal tissues several days post the administration of Truvada (oral tenofovir disoproxil fumarate in combination with emtricitabine) in oral PrEP pharmacokinetic studies.[21] The purpose of selecting this dose is to mimic the situation in which the tissue drug concentration is dramatically decreased from the maximal level, and increasing drug exposure would most likely enhance

microbicide effectiveness. Mice were euthanized 1.5 h after TFV administration. CVL was collected using 60 µL of saline (20 µL saline per wash × 3 times). Blood and tissues were collected as described above for the gel-dosed mice.

To evaluate the safety of MK571 application, in both vaginal and IP routes, mice were administered with non-radioactive TFV gels or solutions, with or without MK571 co-administration. The molar quantity of TFV of the gels and solutions used in these safety evaluations was kept same as in the PK experiments, as detailed in Table 4.2 above. For the vaginal route, mice were administered with 1) 100 µL IP administered saline followed by 20 µL TFV gel; 2) 100 µL IP administered MK571 solution followed by TFV gel; 3) 100 µL IP administered saline followed by (TFV+MK571) gel; 4) 100 µL IP administered saline followed by 20 µL of N-9 gel (4%), as a positive control. The mice were euthanized at 1 h for the vaginal dosing group, or at 1.5 h for the IP dosing group. The uterus, endocervix, ectocervix, vagina and colorectum were collected in the same way as in the PK experiments described above, and fixed in 10% neutral buffered formalin (10% NBF) until further processing for histological staining.

4.2.3 Measurement of radioactivity in mouse samples

The CVL (200 or 60 µL), plasma (100 µL), and tissue (5-50 mg) samples were transferred to scintillation vials. Five hundred µL of Solvable^{TM} tissue lysis buffer (Perkin Elmer) was added into the vials containing the CVL, plasma, or tissues. The vials were then incubated overnight in a 50°C water bath to completely dissolve the tissues. Following this incubation period, the sample vials were taken out and added with 100 µL of H_2O_2 (Fisher Scientific) was added to each sample and incubated at 50°C for 1 hour. The purpose of adding H_2O_2 was to decolorize the

samples in order to eliminate the influence of sample color on subsequent scintillation counting, and 1 hour of 50°C incubation was to completely remove the remaining H_2O_2 which would also affect the activity of scintillation cocktail. The complete removal of H_2O_2 was reflected by the absence of air bubbles arising from the H_2O_2-added vials. After H_2O_2 treatment, the vials were removed from the water bath and cooled to room temperature, followed by the addition of 2.5 mL of ScintiSafe™ Plus cocktail (Fisher Scientific). The vials were vortexed, and placed in a scintillation counter (Perkin Elmer) for the measurement of radioactivity of the CVL/plasma/tissue samples.

4.2.4 Histological evaluation

Hematoxylin & eosin (H & E) staining was performed as previously described,[243] on the genital and colorectal tissues of the mice receiving vaginal gel or IP solution administrations. Briefly, the fixed tissues were embedded into paraffin. The staining was performed on 5 μm sections. Pictures were taken using a Zeiss (Genna, Germany) Axioskop 40 microscope with AxioVision Software.

4.2.5 Measurement of permeability of gel-released TFV across artificial membranes

To evaluate the potential impact of the incorporation of the Mrp4 inhibitor MK571 on TFV release from the formulated gels, the permeability studies were performed using a Franz Cell system as previously described.[243] The 7 mm Franz Cells (Permegear Inc. Hellerstown, PA) were water-jacketed and the temperature was maintained at 37 ± 0.5°C throughout the experiment. The receptor chamber was filled with 5.0 mL PBS (pH 7.4) solution and continuously stirred. The Spectra/Mesh nylon filters (Spectrum Inc.) were sandwiched between donor chamber and

receptor chamber, which yielded a diffusion area of 0.385 cm^2. These nylon filters were used as inert membranes which allow the permeation of small molecules but restrict the permeation of gel matrix. The excessive PBS in the donor chamber was absorbed by cotton tips. The study was initiated by adding 100 µL of TFV gel with or without MK571 into the donor chamber using a 1 mL disposable syringe. Aliquots of 200 µL were removed from the receptor compartment for radioactivity analysis at 1, 15, 30, 45, 60, 120, 180, 240, 300, 360 min after the addition of the gel to the donor chamber. Fresh PBS (pH 7.4) was immediately supplemented to maintain receptor volume and sink conditions. Radioactivity was measured using a Wallac 1409 DSA Liquid Scintillation Counter (Perkin Elmer Life Sciences, Inc., Boston, MA). The amount of TFV was calculated based on the CPM reading. The cumulative amount of TFV in the receptor compartment, as a percentage of the initial TFV amount in the donor compartment, was plotted over time to reflect the permeation profile of TFV through the inert membranes. Apparent permeability coefficient, P_{app}, was calculated as: $P_{app} = \frac{dQ}{dt \cdot A \cdot C_d} \times 60$ ($cm \cdot s^{-1}$), where dQ/dt was the slope of the curve of Q (amount in fmol) versus t (time in minutes), A was the surface area (cm^2) of the inert membrane exposed to the gels, and Cd is the initial amount of TFV in the donor compartment.

4.2.6 Data analysis and statistical methods

The CPM (count per minute) values generated from the scintillation counter were converted to DPM (decay per minute) using the following equation: $DPM = \frac{CPM}{Counting\ efficiency}$. The radioactivity (µCi) of samples was calculated based on Radioactivity (µCi) = DPM / (2.22×10^6). The molar concentrations of ^{3}H-TFV in CVL, plasma, or tissue samples (fmol/µL or fmol/mg

tissue) = CPM/counting efficiency/(2.22×10^6)/specific activity $\times 10^6$. Data were presented as mean ± standard deviation.

For vaginal substrate (TFV) administration: C_{plasma}, C_{CVL}, and C_{tissue} were compared between the three groups using one way analysis of variance (ANOVA) with Bonferroni post hoc test. For IP substrate administration: the following were compared between the two groups: 1) C_{CVL}, C_{plasma} and C_{tissue}, 2) C_{CVL}/C_{plasma} and C_{tissue}/C_{plasma}. The differences were compared using Student's t-test. $P<0.05$ was considered statistically significant, and $p<0.01$ was considered as very significant.

4.3 Results

4.3.1 The effect of MK571 co-administration on the tissue distribution of vaginally administered TFV

The co-administration of MRP transporter inhibitor (MK571) significantly increased the TFV concentration in endocervix, ectocervix and vagina, by up to several fold at the examined time points post vaginal administration of TFV gel. However, the concentrations in plasma, uterus, and colorectum were only slightly increased by the MK571 co-administration (Figure 4.2).

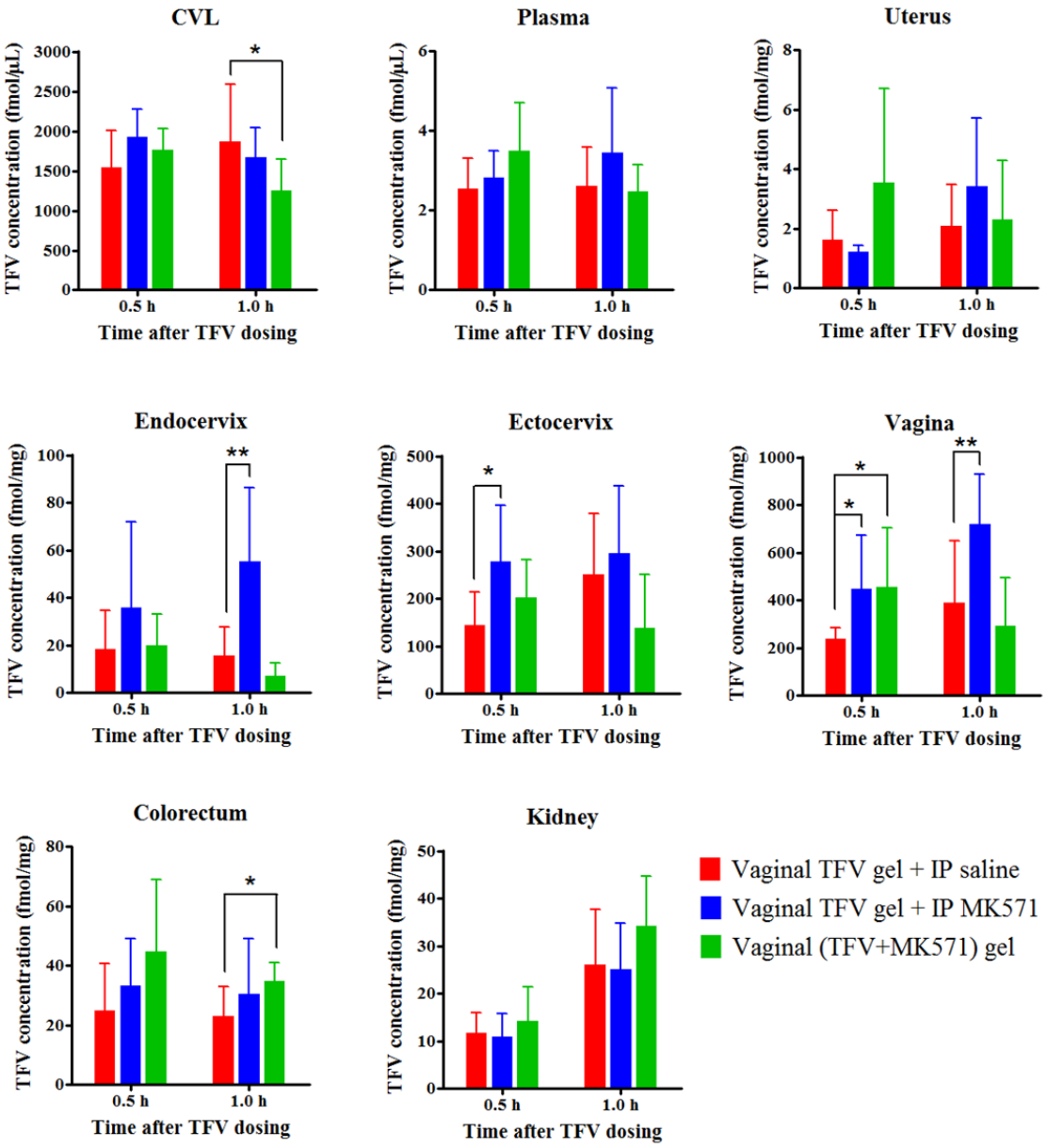


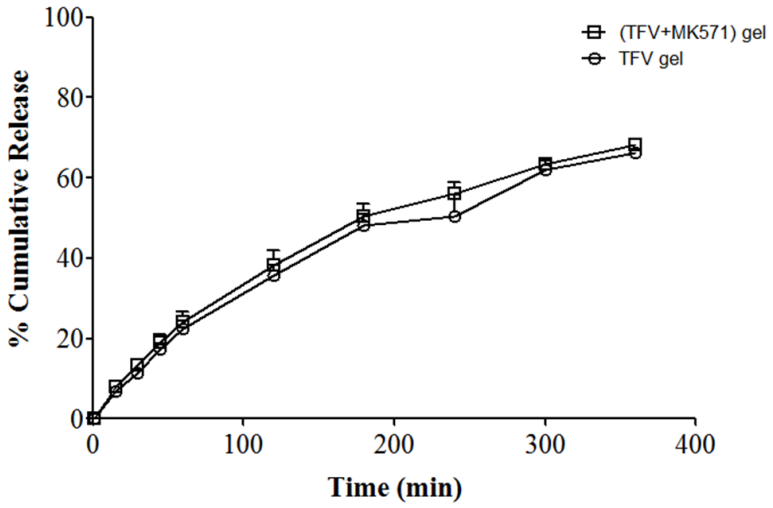
Figure 4.2 The effect of MK571 on tissue distribution of vaginally administered TFV

The effect of MRP transporter inhibitor MK571 on TFV distribution was tested using vaginal gels containing ^{3}H-TFV, with or without the administration of MK571. Mice were euthanized 0.5 h and 1.0 h post TFV gel administration, CVL, plasma, and tissue samples were collected and radioactivity was measured in a scintillation counter. TFV molar concentrations in these samples were plotted for the two time points tested. Red columns, mice were administered IP with saline, followed by vaginal administration of TFV gel; blue columns, mice were administered IP with MK571 solution, followed by vaginal administration of TFV gel; green columns, mice were administered IP with saline, followed by vaginal administration of (TFV+MK571) gel. Data represent mean ± SD. In the group dosed with (TFV+MK571) gel and euthanized at 1.0 h, 4 mice were used. For other groups, 7-13 mice were used. *p<0.05; **, p<0.01.

The incorporation of MK571 into the TFV gel exerted time-dependent effects on TFV distribution. At 0.5 h post gel administration, the TFV concentration in mouse vaginal tissue was significantly higher in the MK571 group. The TFV concentration was slightly increased in plasma, uterus, ectocervix and colorectum, however no statistical significance was observed. The TFV concentration in CVL and endocervix remained unchanged with the addition of MK571 (Figure 4.2). At 1.0 h, the TFV concentration in colorectum was significantly increased, while its CVL concentration was significantly decreased. There was also a decrease in the TFV concentration in endocervix, ectocervix and vagina, but the differences were not statistically significant. The TFV concentration in plasma and uterus remained unchanged with the incorporation of MK571 into the TFV gel (Figure 4.2).

4.3.2 The effect of MK571 incorporation on the release kinetics and permeability of gel-formulated TFV

To examine whether the differential TFV PK profiles of the two kinds of TFV gels were related to the release kinetics of TFV, the Franz Cell permeability experiments were conducted. As shown in Figure 4.4. Compared to the gel containing TFV alone, the incorporation of MK571 into the gel did not have any impact on the in vitro release profile of TFV. The percentages of the cumulative amount released by 30 min and 60 min, which were the two time points employed in the PK experiments described above, were very similar between the two kinds of TFV gels. The permeability coefficient P_{app}, as calculated from the time-dependent TFV concentration appearing in the acceptor chamber of the Franz Cell apparatus, was almost identical for both kinds of gels.



	% Release at 30 min	% Release at 60 min	P_{app} (cm/sec)
TFV gel	11.45 ± 0.93	22.38 ± 2.12	$1.06 \times 10^{-5} \pm 5.67 \times 10^{-7}$
(TFV+MK571) gel	13.46 ± 1.21	24.05 ± 2.64	$1.09 \times 10^{-5} \pm 6.87 \times 10^{-7}$

Figure 4.3 The effect of MK571 incorporation on the release of TFV from formulated gels. Gels with or without MK571 incorporation were applied to the donor chamber of Franz Cell Apparatus, and the TFV concentration in acceptor chamber medium (PBS) was measured to generate the kinetics of TFV release from the gels. P_{app}, permeability coefficient. Results represent mean ± SD from 3 gels in each group.

4.3.3 The effect of vaginal MK571 co-administration on tissue morphology

To evaluate the safety of the co-administration of MK571, H & E staining was performed to examine the morphology of female mouse genital and colorectal tissues after the vaginal administration of TFV gels with or without MK571. N-9 gel was used as a positive control due to its reported damage to the morphology of mouse cervicovaginal tissues.

As shown in Figure 4.4 below, there was no difference in the morphology of tissues, between the mice administered with TFV gels, TFV gel with IP administration of MK571, or the combined (TFV+MK571) gel. After Depo-Provera synchronization, the epithelia in uterus, endocervix, and

ectocervix became single-layer columnar cells, but the intactness of this single layer was maintained. The vaginal epithelia still contained several layers after synchronization, while the colorectal epithelium did not appear to be affected by Depo-Provera. The administration of TFV- and MK571-containing gels and solutions did not cause any discernable damage to the genital and colorectal tracts of female mice (Figure 4.4). On the contrary, the 4% N-9 gel negatively affected the morphological intactness of the epithelia of endocervix, ectocervix, and vagina. Detachment of columnar and squamous epithelial cells from the basal lamina could be readily observed in significant portion of the epithelium-lining regions of these tissues (Figure 4.5). These results demonstrated that the topical application of MK571 did not negatively affect the intact epithelia of the mouse cervicovaginal tissues, which are important natural barriers against sexually transmitted infectious pathogens.[33] In addition, the differential effect of gel-administered MK571 on TFV tissue distribution, compared to the MK571 administered IP, was unlikely due to the altered epithelial intactness upon local administration of MK571.

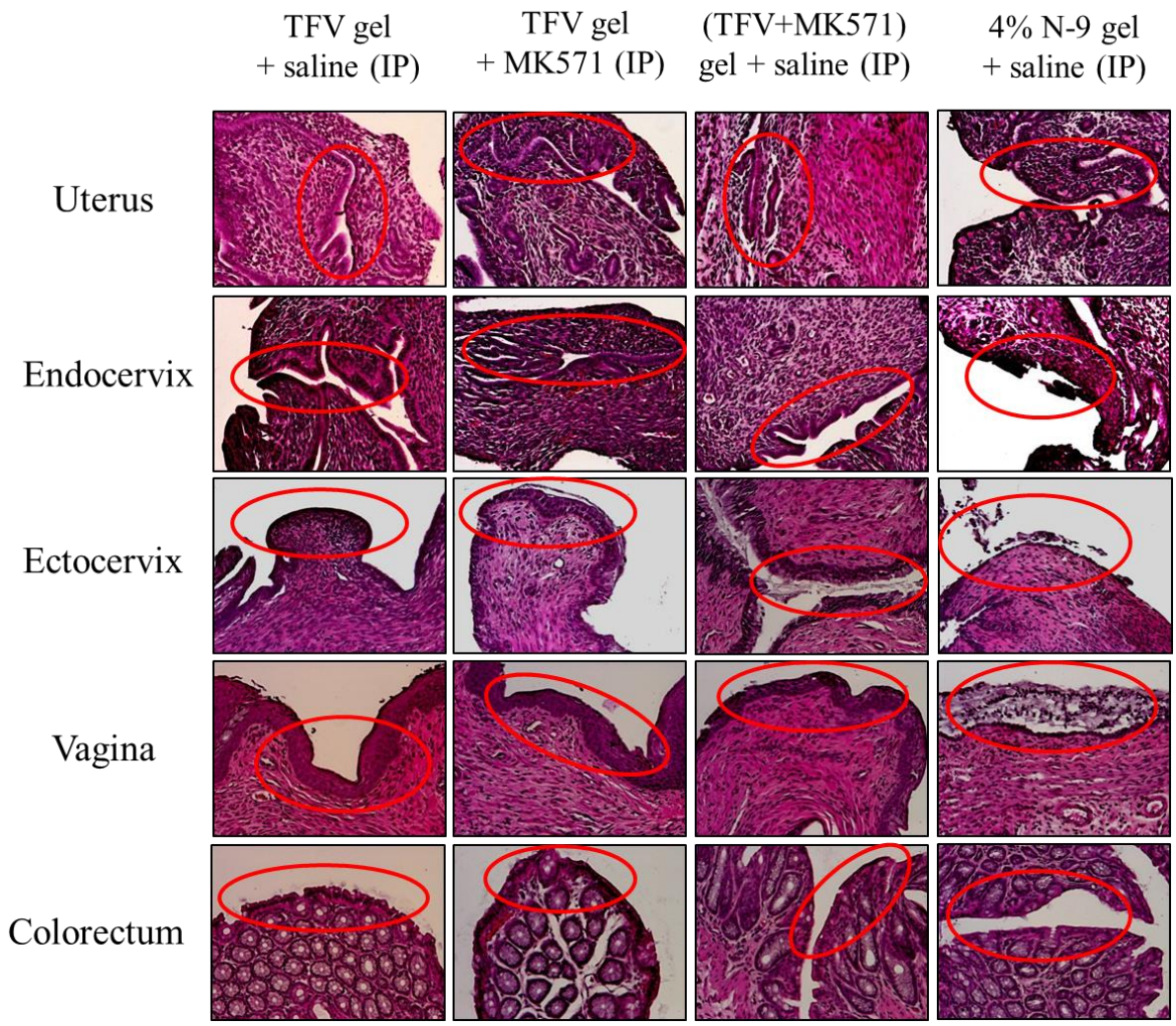


Figure 4.4 H & E staining of mouse cervicovaginal tissues after vaginal administration of gels containing TFV or N-9.
The tissues were collected from mice receiving vaginal gel administrations with IP co-administration of saline and MK571. The gel containing 4% nonoxynol (N-9) was used as positive control since N-9 is a well known disruptor of cervicovaginal epithelial layers. The tissues were rinsed to remove blood or viscous fluid on the surface, fixed in 10% neutral buffered formalin for more than 24 h, and subjected to H & E staining. Red circles highlight the epithelial layers to observe. Magnification is 20 × for all pictures.

4.3.4 The effect of MK571 IP co-administration on the tissue distribution of IP administered TFV

To examine the functional role of Mrp4 transporter in the cervicovaginal/colorectal tissue distribution of systemically administered TFV, mice were given TFV solution through IP route,

with or without MK571 co-administration via the same route 15 min prior to TFV dosing. The TFV concentrations in the three compartments, i.e. cervicovaginal fluid, tissues, and plasma were measured 1.5 h post TFV administration.

As shown in Figure 4.6, compared to its plasma concentration, TFV was preferentially distributed into cervicovaginal tissues and fluid after IP administration. MK571 increased TFV concentration in CVL and vagina (p=0.04), while slightly decreased TFV plasma concentration (-13%).

The vagina/plasma (p=0.03) and colorectum/plasma (p=0.006) ratios were significantly increased by MK571 co-administration, suggesting that the observed increase in vagina and colorectum tissue concentrations found after MK571 application was due to Mrp4 transporter inhibition, thus providing evidence for the functional role of Mrp4 transporter in limiting TFV distribution from blood to these tissues. MK571 significantly increased kidney/plasma ratio by 33% (p=0.002), consistent with published report that the MRP4 transporter limiting kidney tissue TFV accumulation.[84]

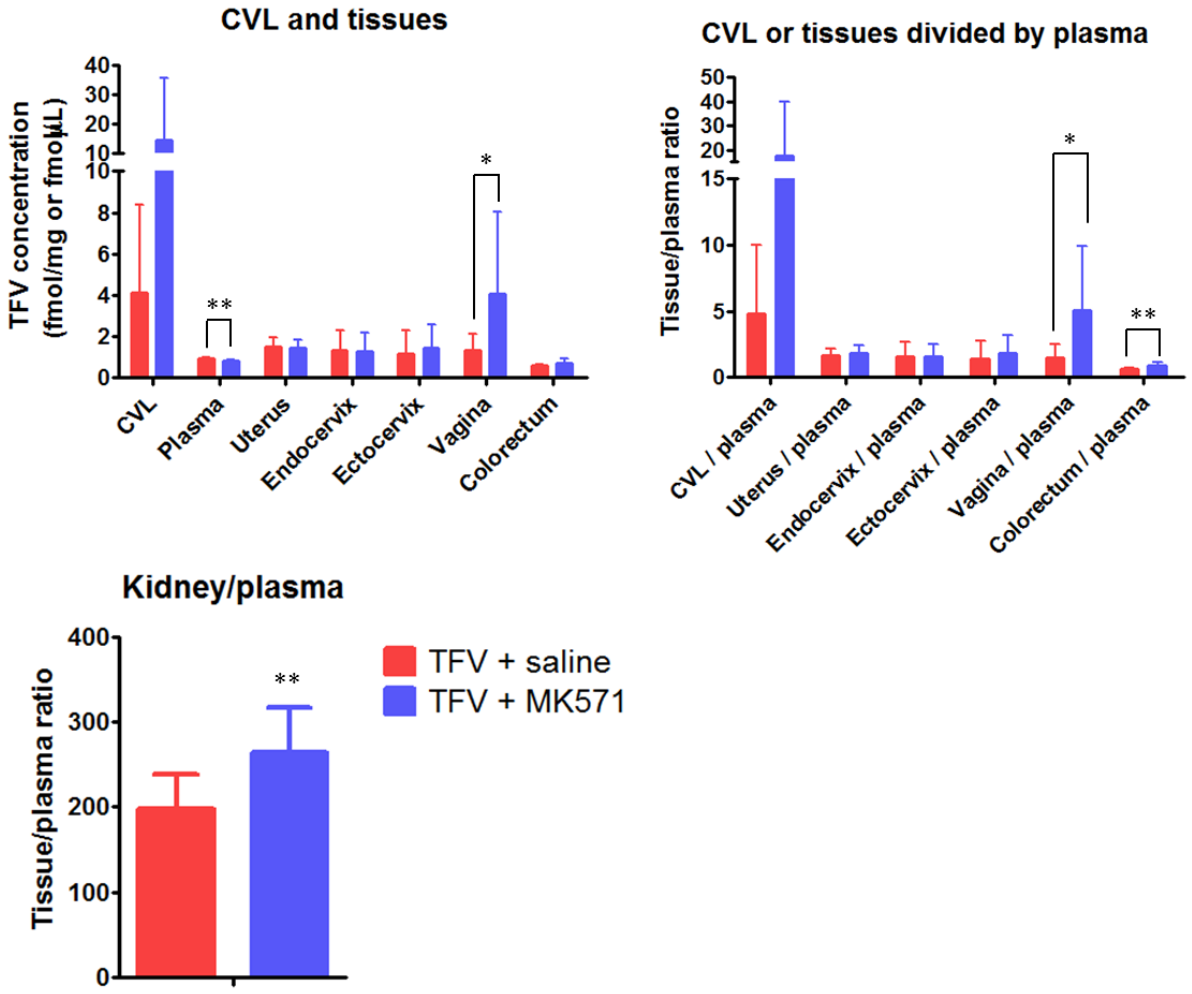


Figure 4.5 Function of Mrp4 transporter in the tissue distribution of systemically administered TFV.
The effect of MRP transporter inhibitor MK571 on TFV distribution was tested. Mice were IP administered with saline (red columns) or MK571 (blue columns), followed by IP administration of ^{3}H-TFV saline solution 15 min later. Mice were euthanized 1.5 h post TFV solution administration. CVL, plasma, and tissue samples were collected and radioactivity was measured in a scintillation counter. TFV molar concentrations in these samples were plotted above. Data represent mean ± SD from 12-13 mice. *p<0.05; **, p<0.01.

4.3.5 The effect of intraperitoneal TFV and MK571 administration on tissue morphology

To examine whether the IP co-administration of MK571 solution had any impact on the morphology of genital and colorectal tissues, H & E staining was performed on these tissues after the dosing of TFV and TFV+MK571 solutions. As shown in Figure 4.7, neither TFV alone

nor the combination with MK571 exerted any effect on tissue morphology and epithelial intactness. N-9 treated tissues were used as positive control and shown in Figure 4.5. TFV solution with or without MK571 did not exert any discernable negative impact on the intactness of cervicovaginal tissues, suggesting that the effect of MK571 on TFV distribution was unlikely due to altered passive permeability which is often a result of compromised tissue intactness.

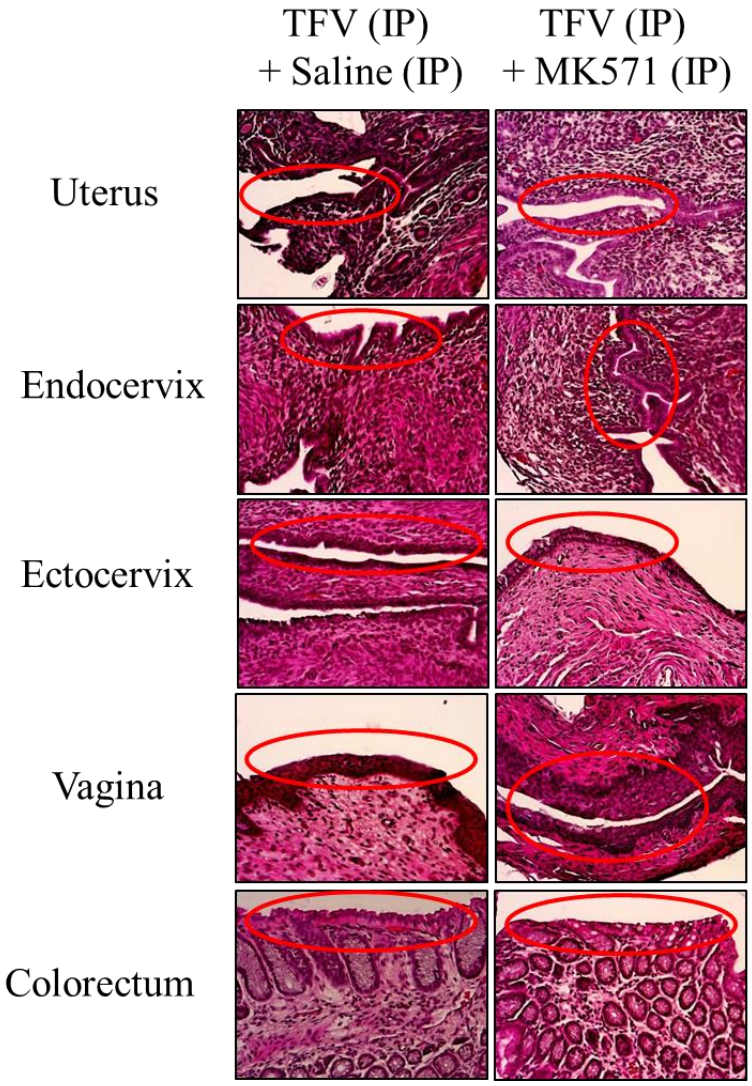


Figure 4.6 H & E staining of mouse cervicovaginal tissues after IP administration of TFV solutions with or without MK571.
The tissues were collected from mice receiving IP TFV solution, with IP co-administration of saline and MK571. The tissues were rinsed to remove blood or viscous fluid on the surface, fixed in 10% neutral buffered formalin for more than 24 h, and subjected to H & E staining. Red circles highlight the epithelial layers to observe. Magnification is 20 × for all pictures.

4.4 Discussion and Conclusion

The studies conducted in this chapter have provided proof of concept that the Mrp4 plays a role in TFV tissue distribution, and the co-administration of MRP inhibitor MK571 could alter the TFV distribution, in a mouse model utilized for vaginal microbicide safety evaluation. TFV is a substrate of two efflux transporters (MRP4, MRP7) and two uptake transporters (OAT1, OAT3). As examined by real-time RT-PCR, OAT1 and OAT3 were found to be undetectable in cervicovaginal and colorectal tissues of human and mice. The GAPDH-normalized mRNA levels of MRP7 were at least 100 fold lower than the Mrp4 level, in the endocervix, ectocervix and vagina of the Depo-Provera synchronized mice (Figure 2.11). The Mrp7 mRNA level in mouse colorectum was more than 10 fold lower than the Mrp4 level (Figure 2.11). Based on the expression levels, Mrp4 is most likely to be the major efflux transporter for TFV, in the cervicovaginal and colorectal tissues of the synchronized mouse model. In addition, the observed effects of MK571 on TFV distribution into cervicovaginal and colorectal tissues were most likely due to the inhibition of Mrp4 transporter in these tissues.

The distribution of TFV in mouse tissues after vaginal administration resembled clinical pharmacokinetics of TFV vaginal gel products, in several aspects. First, mouse vagina concentration of TFV was the highest in all segments of mouse lower genital tract, followed by ectocervix and endocervix, and mouse plasma concentration of TFV was about 100 fold lower compared to the vaginal tissue concentration. This was in line with the clinical observation that vaginal tissue TFV concentration was the highest in human female lower genital tract after gel administration, and human plasma level of TFV was much lower than the tissue level.[21,244] Secondly, mouse colorectum TFV concentration was much lower than the vaginal tissue

concentration but was about 20 fold higher than the plasma concentration. This was similar to the observation that human and macaque colorectal tissue TFV level was lower than vaginal tissue drug level but was much higher than the blood level of TFV after vaginal gel administration.[244,245] Third, mouse kidney TFV concentration was 10-25 fold higher compared to plasma concentration, at 0.5 and 1.0 h after TFV gel administration (Figure 4.2). This is in line with the observation that MRP4 expression in kidney is high, and MRP4 facilitates the efflux of TFV from kidney cells into urine.[84]

The tissue distribution pattern of TFV after IP administration was also comparable to the clinical pharmacokinetics of orally administered tenofovir disoproxil fumarate (TDF). TDF is a prodrug of TFV, and could be converted to TFV and TFV diphosphate metabolites in the body. Although the TFV solution used in the current study is different from TDF, the preferential distribution of TFV into mouse cervicovaginal fluid after IP administration resembled the similar pattern observed after oral TDF administration in humans. At 1.5 h post TFV administration, the average TFV concentration in CVL samples was around 4.11 fmol/μL, which was 5 times higher than plasma concentration. CVL concentration was calculated by dividing the TFV quantity in CVL to 60 μL, which was the volume of saline used to collect the cervicovaginal lavage. The actual fluid volume in mouse vaginal lumen was actually much lower than 60 μL, and TFV concentration in the cervicovaginal fluid should be much higher than the calculated 4.11 fmol/μL. The average TFV concentrations in mouse uterus, endocervix, ectocervix, and vagina were 1.49, 1.33, 1.17, and 1.32 fmol per mg of tissue, respectively, which were not very different from plasma concentration (0.89 fmol/μL). This is in line with the clinical observation that TFV concentration in vaginal tissue did not differ from its blood concentration, and the vaginal fluid

TFV concentration was much higher than blood after oral administration of TDF or Truvada (TDF combined with emtricitabine).[21,244]

For both administration routes (vaginal and IP dosing), wide variability of TFV concentration in female genital tract could be observed between individual mice, at all the time points post administration (0.5 and 1.0 h post vaginal dosing, 1.5 h post IP dosing). Notably, the variability in female mouse reproductive tract was much wider than the variability in mouse plasma and other internal organs such as kidney (Figures 4.2 and 4.4). Similarly, high degree of intra- and inter-subject variability was also observed in human and non-human primate studies of TFV and other antiretroviral drugs such as the viral entry inhibitor maraviroc.[21,41-43,114,244,246-249] This is probably due to the complexity of drug disposition in female genital tract. In the vagina, ectocervix, and endocervix, many factors, such as the epithelial layer thickness, tight junction abundance, vaginal fluid level, transporters and metabolizing enzymes could potentially affect TFV distribution across vaginal lumen, cervicovaginal tissues, and plasma.[12,33,34] These physiologic determinants are regulated by various factors such as hormone status, age, and have large variability between individuals. Therefore, it is not surprising to observe a high level of variability in TFV concentration in the CVL samples and in the lower female genital tract tissues.

In this chapter, the effect of MK571 on TFV concentration was not comparable between different segments of the mouse female genital tract (FGT). In addition, the MK571 exerted differential effects on TFV distribution, when MK571 was co-administered via different routes. The IP co-administration of MK571 solution increased TFV concentration up to 3 fold in endocervix, ectocervix, and vagina, on at least one time point (0.5 or 1.0 h) post TFV

administration. However, the vaginal co-administration of MK571 in the form of gel only resulted in an 80% increase in vaginal tissue TFV concentration at 0.5 h post TFV administration. There was no significant difference in other cervicovaginal tissues and in plasma, but the TFV concentration in CVL at 1.0 h was significantly decreased. It appeared that TFV penetration through the vaginal tissue is greater, when MK571 was given vaginally, resulting in increased TFV concentration in the vaginal tissue at early time point (0.5 h), and decreased TFV content remaining in the gel at later time point (1.0 h), but not in other tissues.

One possibility underlying these differential effects of MK571 could be that the incorporation of MK571 into the TFV gel altered TFV release kinetics. However, the release profile of TFV was not altered by MK571 incorporation, as examined using Franz Cell apparatus which is the standard USP method of testing the drug release from semisolid dosage forms. The two dosing routes of MK571 did not cause differential impacts on epithelial morphology, compared to the groups dosed with the universal placebo gel or dosed with TFV gel containing no MK571. Therefore, the differential effects of MK571 were not caused by the differences in TFV release or epithelial integrity, when MK571 was dosed via different routes.

Another possible explanation for these differential effects is that different dosing routes of MK571 resulted in differential levels of MK571 in the tissues examined. When MK571 was given IP, it can be distributed into different segments of the lower female genital tract, to similar degrees due to relatively high blood perfusion into reproductive tissues. However, in the scenario of vaginal administration, the primary site of MK571 absorption is vagina, and the MK571 exposure in ectocervix and endocervix was much lower, resulting in a more obvious effect of

MK571 in the vaginal tissue but not in ectocervix and endocervix. If this is the case, the changes in TFV concentration in mouse vagina, CVL and plasma samples can be explained accordingly. It is possible that when MK571 was given intravaginally in the form of gel, it facilitated TFV entry into vaginal tissue, and increased the TFV concentration in vaginal tissue and plasma at early time point (0.5 h). Since TFV penetrated more quickly, less TFV gel remained in the vaginal lumen at the later time point (1.0 h), resulting in reduced amount of TFV in the CVL samples at the 1.0 h time point, and reduced vaginal tissue concentration at 1.0 h. Therefore, the experiments using IP administered MK571 provided the proof of concept for Mrp4 function in the tissue distribution of vaginally administered TFV, however the feasibility of incorporating a transporter inhibitor to increase the tissue distribution of gel-released TFV will need further testing using MK571 at higher concentrations.

Several future studies will be needed to address the potential issues associated with MK571 concentration in mouse tissues. Ideally, MK571 concentration should be measured to link the observed changes in TFV PK to the inhibitory effect of MK571. However, due to the lack of a validated method of quantifying MK571 tissue concentration, MK571 concentration was not measured in the tissues where it was supposed to exert inhibitory effect. IC_{50} values of MK571 for the MRP4 transporter were reported to be at μM level.[250,251] In order for a chemical inhibitor to be effective in reversing tissue efflux transporters, the tissue concentration of this inhibitor should be at least several fold higher than its *in vitro* IC_{50}. The time course of MK571 concentration in target tissues should also be considered when selecting the time points of *in vivo* sample collection. Based on previously published *in vivo* studies which used MK571 to study Mrp4 transporter in tissues including mucosal tissues such as mouse lung,[240-242] we suspected

that the MK571 concentration in our study was sufficient at early time points (0.5 and 1.0 h in the vaginal TFV dosing experiments, 1.5 h in the IP TFV dosing experiments). Future studies will need to address this shortcoming. These studies could include the development of an analytical method for MK571 quantification, and measurement of its tissue concentration after the IP or vaginal co-administration to mice. In addition, the effect of MK571 dose on TFV PK could be explored to provide more evidence on the functional role of Mrp4 in TFV tissue exposure. If the observed changes in TFV distribution was indeed due to MK571 inhibition on Mrp4, then the dose-dependent effect of MK571 may be observed. Moreover, different strategies of transporter inhibition could be incorporated into future studies to confirm the role of Mrp4 in TFV PK. Nonsteroidal anti-inflammatory drugs, such as ibuprofen, are also potent inhibitors of MRP4 transporter.[89,90] Abcc4 (Mrp4) knockout mice may also be employed to eliminate all the issues with chemical transporter inhibitors. Overall, these future studies will address the issues associated with the tissue concentration of MK571 and other chemical inhibitors, and will validate the findings on Mrp4 function described in this chapter.

In addition, future studies are warranted to further evaluate the functional role of Mrp4 in TFV exposure in cervicovaginal and colorectal tissues. First, The experiments conducted in this chapter have examined Mrp4 function in TFV distribution into three physiologic compartments (CVL, tissues, plasma), at 1 or 2 selected time points after TFV administration. However, since the area under concentration-time curve (AUC) is a more accurate measurement of drug exposure, more time points need be tested in future studies, in order to understand the role of Mrp4 in the AUC of TFV in tissues and fluids relevant to HIV-1 transmission. Secondly, Future pharmacokinetic studies with more doses of substrates will provide more detailed information of

the Mrp4 transporter function in cervicovaginal tissues. In this chapter, one dose was tested, which was below the TFV dose used in clinical testing in order to avoid the possible saturation of Mrp4 transporter. Higher doses will need to be tested to account for the possible saturation of efflux transporters by the substrate drug.

Moreover, the Mrp4 expression level changes in response to mouse estrous cycle, the estrogen-stimulating hormone PMSG, and Depo-Provera. The role of Mrp4 in tissue TFV distribution may be different under these physiologic/pharmacologic conditions. Therefore, Future PK studies under these conditions will provide a comprehensive picture of Mrp4 function and inhibitor effectiveness in the context that mimics clinical use of PrEP drugs.

An additional focus of future PK studies will be to examine the time course of metabolite formation and disposition within the three physiologic compartments (cervicovaginal fluid, cervicovaginal tissues, and blood). In this chapter, the ^{3}H-labeled TFV was used as Mrp4 substrate, the measured radioactivity reflected the total drug amount in the samples, and the concentration of TFV metabolites remained unknown. Although the formation of TFV phosphate metabolites is negligible compared to the parent drug (<5% of TFV), the information of metabolite exposure in tissues is actually more important for estimating efficacy, because the TFV diphosphate metabolite (TFV-DP) is the only active form of TFV. Therefore, future studies could utilize non-radiolabeled TFV as substrate, and adopt established LC-MS/MS method to quantify both TFV and TFV-DP in administered mouse tissue samples.[252]

Although the mouse model represents a convenient *in vivo* tool for proof-of-concept studies, it is not without limitations. Future studies of transporter function using more biologically relevant models or the analyses of human data could address these limitations. Mouse transporters may differ from human counterparts with respect to the specificity and/or affinity to the substrate. In addition, some parts of mouse reproductive tract, such as the uterus, differs from human tissues in terms of morphology and physiology. These interspecies differences may cause potential issues to consider when using the mouse results to predict the function of transporters and feasibility of transporter inhibitors in humans studies. Macaques possess female reproductive tract and colorectal tissue with similar anatomy and philology to human counterparts. Therefore, compared to the mouse model, the macaque model is considered to be more biologically relevant, in PrEP research and other studies related to female genital tract and/or colorectal tissues. In addition, macaques can be infected by simian immunodeficiency virus (SIV) or the chimeric SHIV generated from the combination of HIV and SIV, thus making them a good model for simultaneous evaluation of PK and efficacy of HIV PrEP drug products.[198,247,248] The utilization of the macaque model in future functional study of cervicovaginal and colorectal transporters could reveal whether the observed changes in tissue drug concentration correlates with drug efficacy in HIV prevention. In addition to animal models, the analysis of human PK data and tissue samples obtained from previous PrEP clinical studies testing oral products and topical microbicides may provide direct evidence for the function of cervicovaginal tissue transporters in humans. Such evidence could be obtained by analyzing the correlations between the clinical PK data (substrate antiretroviral drug exposure in tissues relevant to HIV transmission) and transporter expression level or genotype. Such analyses may also provide information regarding

the regulation of transporter expression/activity in cervicovaginal and colorectal tissues under the influence of hormones, contraceptives, and inflammation.

Lastly, the function of other highly expressed transporters also warrants further testing. As revealed by examinations conducted in Chapters 2 and 3, P-gp, BCRP, MRP5, and MRP7 are also highly expressed efflux transporters in human cervicovaginal and colorectal tissues. In mouse tissues, these transporters, except Mrp7, are also expressed at high levels. The IHC staining confirmed the critical localization of mouse P-gp (Abcb1a/Abcb1b) and Bcrp in the cell types that may control drug disposition in tissues (epithelial cells, vascular endothelial cells). The function of P-gp and BCRP could be the priority in the list of transporters to be tested in the functional studies, and the mouse model appeared to be the model of choice due to the high level of expression of these two transporters.

In conclusion, the studies described in this chapter have provided preliminary evidence to demonstrate Mrp4 function in TFV distribution into cervicovaginal and colorectal tissues. The data also suggested that the co-administration of MRP inhibitor could alter TFV distribution, at least when the inhibitor is dosed at proper level via appropriate route. However, it should be noted that the adherence is the key to the drug exposure and efficacy for once daily PrEP products. The functional role of MRP4 transporter and the effectiveness of MRP4 inhibitor could be observed only when the adherence is kept at high level. In the CAPRISA 004 study, participants with vaginal fluid TFV concentration higher than 1000 ng/mL had more than 3 times lower HIV acquisition rate compared to the participants whose vaginal TFV concentration was lower than 1000 ng/mL (infection rate 3.7% versus 12.3%).[13,21] Participants had highly variable

TFV concentration in vaginal fluid, from below the limit of detection (<1 ng/mL) to nearly 10^7 ng/mL. The major cause for this large inter-individual variability has been identified to be low adherence to the gel products, and the efficacy differed significantly among participants with different degrees of adherence.[13] Seventy six percent of the 335 participants has TFV concentration below 1000 ng/mL. Only 12.5% of participants had fluid concentration between 11-1000 ng/mL, and 63% of participants had fluid concentration below 10 ng/mL. In this study, TFV concentration in cervicovaginal tissues was increased by up to 3-4 fold, when MK571 was co-administered. If this tend can be observed in humans, and if the inhibitor approach could be used in the participants of the CAPRISA 004 PK study, then only a small number of participants with 11-1000 ng/mL vaginal concentration might be able to achieve the effective TFV concentration (1000 ng/mL) with the help of the inhibitor approach. Apparently, for the low-adherent participants, the co-administration of MRP transporter inhibitor does not appear to be an effective approach to increase the effectiveness of gel products in those people who do not use the once-daily products. In these scenarios, the most effective strategy could be the utilization of sustained release formulations, such as the vaginal ring or long-acting injectable.[26,29-32,35,36,212] Once the problem of product adherence is resolved (by increasing patient adherence or changing the dosage form to long-acting injectables or intravaginal rings), the information on transporters may be utilized to interpret intra- and inter-individual variability in PK/PD, and the utilization of transporter inhibitors will likely enhance drug exposure and/or reduce the drug load of PrEP products.

The information generated from this chapter, as well as future studies that would examine more time points, more drugs, and more transporters, will facilitate PrEP optimization in multiple

aspects. First, this information is useful for the optimization of PrEP drug candidates. If the transporters play a significant role in drug distribution into the cervicovaginal tissues, then non-substrate drugs may be better candidates than transporter substrates, if all other attributes are similar. Alternatively the substrates could be chemically modified to remove transporter binding moieties while at the same time retain the antiviral activity. In addition, the information on transporter function will facilitate the optimization of PrEP product formulations and inform the design of combination PrEP products. Some antiretroviral drugs, such as protease inhibitors, are potent inhibitors of efflux transporters through competitive binding.[49] Some pharmaceutical excipients, such as pluronic P85, polyethylene glycol (PEG) 300, and cyclodextrins (CDs) have been reported to inhibit ATP-binding cassette (ABC) transporters including P-gp, BCRP, and MRPs.[59] The efflux activity of ABC transporters requires the intracellular availability of ATP, and certain degree of plasma membrane fluidity to conduct conformational changes during the efflux process. The transporter-inhibiting excipients could temporarily inhibit intracellular ATPase activity and decrease the cell membrane fluidity, thus reducing the intracellular ATP production and restricting the conformational change of transporters at plasma membrane.[59] In addition to chemical inhibitors, nano-sized drug delivery systems have been reported to cross plasma membrane via routes different from free drug, and represent an alternative strategy to circumvent the membrane transporter-mediated efflux.[208] Utilization of these strategies to overcome transporter efflux may increase drug exposure and efficacy in HIV-1 prevention.

5 DISCUSSION OF MAJOR FINDINGS AND FUTURE DIRECTIONS

5.1 Major findings, implications, and limitations

More than two million new HIV-1 infections occur each year, and there is an urgent need to curtail this global pandemic. Since a large portion of the new infections are due to poorly protected sexual intercourse, there is a great need for drug products that can effectively prevent the sexual transmission of HIV-1. Although antiretroviral-based topical and oral PrEP products have shown efficacy in some clinical trials, other studies have achieved inconsistent results, and strategies are needed to enhance the effectiveness of PrEP products. Since the antiretroviral drug exposure in the cervicovaginal and colorectal tissues is critical for PrEP effectiveness, and given that the efflux and uptake transporters are key regulators of tissue drug exposure, a greater understanding of the expression and function of drug transporters in tissues and cells relevant to HIV-1 sexual transmission is urgently needed. The studies presented provide critical information regarding the expression, regulation, and function of transporters in human cervicovaginal and colorectal tissues, as well as in the animal models and cell lines utilized in PrEP product screening. This information is valuable for the development of novel strategies that can increase tissue drug exposure, and will ultimately lead to the design of more effective drug products for HIV-1 prevention.

The work conducted in this dissertation was based on the hypothesis that multiple efflux and uptake transporters are positively expressed in the cervicovaginal tissues and play a significant role in tissue drug exposure, which can be modulated by transporter inhibitors. The studies

conducted to address this hypothesis included examination of the mRNA and protein expression of multiple transporters in humans, animal models, and cell lines, under the influence of menstrual cycle, exogenous hormones/contraceptives, and proinflammatory cytokines, as well as the investigation of Mrp4 function in a mouse model. The major findings of the dissertation are summarized below.

5.1.1 mRNA expression of multiple transporters in tissues and cells relevant to HIV-1 transmission

Previously published studies indicated that some efflux and uptake transporters were expressed in cervicovaginal and colorectal tissues, as well as in HIV-1 target immune cells. However, there was a lack of systematic characterization of transporter mRNA and protein expression in these tissues and cells, under the conditions commonly encountered by PrEP participants. These conditions included menstrual cycle, exogenous hormones, hormonal contraceptives, and local tissue inflammation. This information is important for understanding the dynamics of transporter expression in these tissue sites, and is needed for experimental design and data interpretation of further studies concerning cervicovaginal and colorectal transporters.

To fill this knowledge gap, the studies conducted in Chapter 2 of this dissertation first examined the mRNA expression of 9 efflux transporters and 13 uptake transporters which are involved in the transport of antiretrovirals and other therapeutic drugs, using conventional RT-PCR. As hypothesized, 5 efflux transporters (P-gp, BCRP, MRP4, MRP5, MRP7) and 4 uptake transporters (OAT2, OCT2, ENT1, and OATP-D) were found to be moderately to highly expressed in the human ectocervix and vagina, compared to their levels in liver.

The real-time PCR was conducted to validate the positive mRNA expression of the efflux and uptake transporters most relevant to antiretroviral drugs (P-gp, BCRP, MRP4, 5, 7, OAT 2, OCT2, ENT1), in human cervicovaginal and colorectal tissues. P-gp, BCRP, MRP4, 5, 7, and ENT1 were found to be expressed in cervicovaginal and colorectal tissues at levels comparable to or higher than the liver. OAT2 and OCT2 were not detectable using real-time PCR in human tissues, presumably due to the difference in the processing method of extracted RNA. The genomic DNA was removed in real-time PCR experiments, while it was not removed during the conventional RT-PCR. The primers designed for these transporters could amplify from both genomic DNA and mRNA. Therefore, the positively expressed transporters in human cervicovaginal and colorectal tissues were P-gp, BCRP, MRP4, MRP5, MRP7, and ENT1.

Based on the mRNA expression profile of the transporters examined, 16 antiretroviral drugs could potentially be affected by the positively expressed transporters, as discussed in Chapter 2 in detail. Taking the Biopharmaceutics Classification System (BCS) into consideration, the antiretroviral drugs that are potentially affected by the positively expressed transporters in cervicovaginal and/or colorectal tract include nevirapine (MRP7), atazanavir (P-gp), ritonavir (P-gp), amprenavir (P-gp), lopinavir (P-gp), saquinavir (P-gp), darunavir (P-gp), indinavir (P-gp), tipranavir (P-gp), nelfinavir (P-gp) and raltegravir (P-gp), maraviroc (P-gp), lamivudine (BCRP), tenofovir DF (P-gp), tenofovir (MRP4, MRP7), didanosine (BCRP). The antiretroviral drugs that are potentially affected by positively expressed uptake transporter include didanosine (ENT1).

The mRNA expression of these transporters was also examined in the tissues of pigtailed macaques, rabbits, Depo-Provera synchronized mice, as well as three cervicovaginal epithelial cell lines (End1/E6E7, Ect1/E6E7, VK2/E6E7 derived from human endocervix, ectocervix, and vagina) and a T cell line (PM1). These animal models and cell lines are used for the efficacy and/or safety testing of PrEP products especially topical microbicides. The results have shown positive expression of the transporters being tested, in animal tissues and cell lines. The absence of OAT1 and OAT3 in all the tissues and cells examined suggested that these two uptake transporters were not likely to paly a functional role in drug pharmacokinetics in human cervicovaginal and colorectal tissues, as well as in the tissues and cell lines used in PrEP drug candidate evaluation.

A multi-species comparison has been conducted to evaluate the similarity in the expression profile of P-gp, BCRP, MRPs4, 5, 7, between human tissues and animal tissues/cell models. The transporter mRNA expression of pigtailed macaque tissues is most similar to that of human tissues. The Mrp4 levels in rabbit cervicovaginal and colorectal tissues were more than 15-fold higher compared to human tissues, and caution will be needed when interpreting the drug concentration and safety data from the rabbit model. The human-rabbit differences for other transporters were within ± 5 fold. The difference in mRNA levels of P-gp, BCRP, Mrp4 and Mrp5 in mouse tissues were within ± 5 fold from those levels in human, but the level of Mrp7 in all mouse tissues was more than 15-fold higher than the Mrp7 level in corresponding human tissues. In the three cervicovaginal cell lines (End1/E6E7, Ect1/E6E7, VK2/E6E7), P-gp and BCRP mRNA levels were more than 15-fold lower than the levels in human endocervix,

ectocervix and vagina. The levels of MRP4, 5, and 7 were no more than 5-fold lower in the cell lines, compared to corresponding human tissues.

Sex steroid hormones, hormonal contraceptives and proinflammatory cytokines have been demonstrated to influence transporter expression and activity in tissues such as liver. The female PrEP product users have cyclic changes of sex steroid hormones in the cervicovaginal tissues, and the hormone levels are significantly altered after menopause. In addition, a significant portion of users take hormonal contraceptives such as Depo-Provera (long-lasting injectable suspension of MPA), and have increased levels of proinflammatory cytokines such as IL1β and IL8 in the genital tract which is caused by bacterial or fungal vaginosis. Therefore, it is necessary to examine the effect of menstrual cycle, menopause, contraceptives, and proinflammatory cytokines on transporter expression in the cervicovaginal tissues. To address this, the effect of menopause was studied by comparing transporter mRNA levels in pre- and postmenopausal human ectocervix; the effects of MPA and P4, as well as IL1β and IL8 on efflux transporter mRNA expression were examined using End1/E6E7, VK2/E6E7, PM1 cell lines; the effects of menstrual cycle, PMSG, and Depo-Provera were examined in a mouse model. Five transporters, P-gp, BCRP, MRP4, 5, 7 were examined. In cell lines, the effects were found to depend on the type of transporter and type of cell. The contraceptives and cytokines appeared to regulate transporter mRNA expression toward opposite directions in different cells (End1/E6E7/VK2/E6E7 vs. PM1). In mice, the general tendency in cervicovaginal tissues was more obvious than in other tissue types: transporter mRNA levels were higher at diestrus stage compared to estrus stage, during the natural estrous cycle, which mimicked the secretory phase and proliferative phase in human menstrual cycle. In addition, the Depo-Provera treatment

resulted in significant up-regulation of the mRNA levels of several cervicovaginal transporters, compared to the natural estrous cycle, while PMSG induced the opposite effect by reducing mRNA levels.

Understanding the mechanisms underlying the observed transporter regulation by hormones and cytokines in cervicovaginal and colorectal tissues is important for the prediction of other factors that can regulate transporters through similar mechanisms. Nuclear receptors (NRs) are transcriptional factors that mediate hormone- and cytokine-induced regulation of transporters. As the first step toward elucidating the transporter regulation mechanisms in cervicovaginal and colorectal tissues, the mRNA expression of 23 nuclear receptors (NRs) was examined in these tissues of humans, macaques, and mice. The real-time PCR showed abundant expression of NRs that can bind sex steroid hormones (e.g. ER-α, PR) or proinflammatory cytokines (e.g. Nrf2, AhR), while negligible expression of typical xenobiotic-sensing NRs (e.g. PXR). The expression profile of these NRs in macaques and mice showed similar tendency as compared to human, with absent or very low levels of PXR mRNA expression and high levels of hormone-responsive NRs.

5.1.2 Protein expression of three efflux transporters in cervicovaginal and colorectal tissues

Since mRNA levels do not necessarily correlate with protein expression, the protein abundance and localization of three efflux transporters (P-gp, BCRP, MRP4) were examined in the tissues of humans, macaques, and mice, using IHC staining.

As described in Chapter 3, in human tissues, P-gp, BCRP, MRP4 were found to be positively expressed, in multiple cell types within the cervicovaginal tissues, including columnar epithelial cells, stratified squamous epithelial cells, and vascular endothelial cells. Transporter protein

abundance and localization depended on the type of transporter and type of tissue examined. Compared to the premenopausal human ectocervix, the postmenopausal ectocervical tissues appeared to have more diffuse staining of P-gp, BCRP and MRP4 in the epithelial cells, however the vascular wall in the epithelium and stroma region remained densely stained for all the three transporters. It is unclear whether the transporter activity in postmenopausal tissues will be different from that of premenopausal tissues, and whether this will affect drug exposure in the tissues. Compared to premenopausal human tissues, the transporter protein expression in macaque tissues were very similar.

The protein abundance of the three transporters appeared to be in line with their mRNA levels, in mouse cervicovaginal tissues collected at two different stages of natural estrous cycle, or after synchronization using PMSG/Depo-Provera. Consistent with the tissue- and transporter-specific mRNA levels, each transporter was differentially expressed in mouse endocervix, ectocervix and vagina, at protein levels. The expression patterns of P-gp, BCRP, and MRP4 at mRNA and protein levels in Depo-Provera treated mice were generally comparable to those patterns in human tissues.

5.1.3 Transporter function in tissue distribution of PrEP drugs

Since tenofovir (TFV) has been extensively tested in vaginal and oral PrEP trials, as a single agent as well as in combination regimens, it is necessary to test the role of transporters in the distribution of TFV into tissues important for HIV-1 sexual transmission. MRP4 was found to be positively expressed in the cervicovaginal tissues at levels comparable to liver in human and animal models tested, and its expression level was markedly up-regulated during the use of

Depo-Provera, as demonstrated in the mouse model. Under the treatment of Depo-Provera, it is highly possible that MRP4 plays a significant role in TFV tissue distribution, since its expression is higher, while the epithelial thickness and tight junction abundance are reduced compared to untreated status. The Depo-Provera synchronized mice were used by some researchers to evaluate the safety of vaginally administered products for the prevention of sexually transmitted infections. Since a large percentage of PrEP participants take contraceptives, and Depo-Provera is the most widely used contraceptive in Sub-Saharan Africa where the rate of HIV-1 sexual transmission is highest, the study using this mouse model could enable better understanding of Mrp4 function in the cervicovaginal and colorectal tissues of PrEP participants who take Depo-Provera. Based on these considerations, the role of Mrp4 transporter in cervicovaginal and colorectal distribution of TFV was studied in this model, in Chapter 4.

When TFV was administered as a vaginal gel, the intraperitoneal (IP) co-administration of Mrp4 inhibitor MK571 significantly increased TFV concentrations in mouse endocervix, ectocervix, and vagina, at one or both time points (0.5 h or 1.0 h) after TFV administration. The TFV concentrations in CVL, uterus, plasma, and colorectum were not significantly altered by MK571, at either time point tested.

When MK571 was formulated into the TFV gel, it exerted time-dependent effect on tissue concentration of TFV. At 0.5 h, it significantly increased the vaginal tissue concentration and did not alter TFV concentration in other tissues or plasma. At 1.0 h, MK571 incorporation increased the colorectal tissue concentration, as well as the remaining TFV content in vaginal lumen, as reflected by the increased CVL concentration. The differential effects of MK571 on TFV

distribution were not due to the alteration in release kinetics of TFV from the two kinds of formulated gels, as demonstrated by the unchanged amount of TFV cumulative release at 0.5 and 1.0 h, and unchanged TFV permeability coefficients. In addition, the differential effects of the gel-formulated MK571 was not likely caused by increased impact on epithelial intactness, as demonstrated by the unchanged morphology of cervicovaginal epithelia after IP or vaginal co-administration of MK571. Another possibility underlying the differential effects of MK571 was the differential MK571 concentrations achieved when being dosed via different routes (vaginal vs. IP), as discussed in Chapter 4. This possibility warrants further testing using TFV gels with increased doses of MK571, to delineate the effect of topically applied transporter inhibitor on TFV tissue distribution.

For the systemically (IP) administered TFV, the co-administration of MK571 (IP) resulted in a 3.5-fold yet not significant increase in the TFV concentration in CVL. Further, MK571 significantly increased the vagina/plasma and colorectum/plasma ratios, suggesting that the increase in vaginal and colorectal tissue concentrations was due to the inhibition of Mrp4 transporter in local tissue sites not the increased TFV availability in blood.

These proof-of-concept studies using TFV gel and TFV solution in Chapter 4 provide preliminary evidence of Mrp4 function in TFV distribution into cervicovaginal and colorectal tissues. In addition, these results demonstrate the feasibility of using Mrp4 inhibitor to increase TFV distribution into the cervicovaginal fluid, cervicovaginal tissues, or colorectum. However, it should be noted that the effect of Mrp4 inhibitor on TFV distribution appeared to depend on the route of TFV and inhibitor administration, and further testing is needed as proposed below.

5.1.4 Implications on antiretroviral-based HIV-1 prevention

The results generated from the studies in this dissertation have wide implications for the development and optimization of drug products for HIV-1 prevention. First, the positive expression of multiple efflux transporters at mRNA and protein levels, suggest that these transporters may function to limit the distribution of their substrate drugs into female genital tract and colorectal tissues. Notably, the efflux transporters are expressed at relatively higher levels, compared to the uptake transporters and CYP enzymes, suggesting that the efflux transporters may be more important regulators of drug exposure in the cervicovaginal tissues, compared to uptake transporters and CYP enzymes.

Secondly, the observation that MK571 co-administration could increase the cervicovaginal tissue drug exposure of both vaginally and systemically administered TFV suggested the Mrp4 transporter was functional in cervicovaginal tissues. Although further studies are needed to validate the functional role of Mrp4 and other efflux transporters, these results serve as the proof of concept that TFV tissue distribution could be enhanced by pharmacologic inhibition of the Mrp4 transporter.

In addition, the effect of estrous cycle, contraceptives, hormones, and inflammation-related cytokines on cervicovaginal/colorectal transporter expression suggested that the expression of transporters in these tissues can be variable under different pathophysiological conditions. Caution should be implemented when designing experiments and interpreting data when they involve drugs which are potential substrates of transporters present in cervicovaginal or colorectal tissues. Transporters present in these tissues may have varying degrees of contribution

to the overall pharmacokinetics of drugs, which is a potential source of inter-individual and intra-individual variability in drug PK profile in those tissues. The expression profiling of 23 nuclear receptors in human, macaque and mouse tissues suggested that multiple NRs were potential mediators of the observed effects of hormones, contraceptives, and cytokines on transporter expression. In addition, the absence of PXR expression in cervicovaginal and colorectal tissues suggested that the PXR receptor is unlikely to be an important mediator of transcriptional regulation of cervicovaginal or colorectal transporters, and transporters in these tissues are unlikely to be regulated by many therapeutic drugs that can bind and activate PXR.

Multi-species comparisons in transporter expression and localization have implications on the rational selection of research models, for the studies of transporter function and safety/efficacy evaluations of microbicide products. In addition, the comparisons have implications on the validity of models used in PrEP testing, especially when the tested drug is a substrate of one or more transporters highly expressed and functional in the tissues.

5.1.5 Limitations

The major limitations of the studies conducted in this dissertation are listed below:

There are several limitations related to this study of transporter expression. In Chapter 2, when assessing the transporter expression in immune cells, those immune cells residing in the cervicovaginal and colorectal tissues are the primary target of HIV-1 particles. Although the transporter expression in blood-derived immune cells have been reported and the expression in a T cell line (PM1) has been characterized in this dissertation (Chapter 2), it remains unknown

whether the expression and activity of major transporters will be altered when the immune cells migrate into tissues. In Chapters 2 and 3, when testing the effect of hormones and contraceptives and inflammation, the effect of concentration remains unknown. In addition, when examining the transporter expression in colorectal tissues, it remains unknown whether the gender will affect the expression level and function. In this study, the colorectal tissue levels of transporters were examined only in female tissues but not in male tissues. However, men who have sex with men (MSM) are especially vulnerable to the transmission of HIV-1 through anal intercourse, and colorectal transporter expression needs to be evaluated for this subpopulation.

In Chapter 4, the effect of MK571 on TFV distribution was studied at one (intraperitoneal TFV dosing) or two (vaginal TFV dosing) time points post TFV dosing. The results provide evidence for the effect of Mrp4 inhibition on TFV distribution at these time points. However, the area under the concentration-time curve (AUC) is a better indicator of drug exposure compared to single time point measurements. AUC data will enable a more comprehensive understanding of tissue drug exposure over the entire period of TFV product use.

5.2 Proposed future studies

Below is a list of proposed future studies, which could address the limitations discussed above and extend the studies to achieve further understanding of transporter expression, regulation and function in the tissues and cells relevant to HIV-1 sexual transmission.

1. Examine the expression profile of transporters in the HIV-1 target immune cells associated with cervicovaginal and colorectal tissues. Due to the paucity of healthy human tissues, the

quantity of purified immune cells from tissues may be insufficient for subsequent studies which aim to examine their function. However, by comparing the expression levels between these tissue-associated cells and blood-derived primary immune cells (e.g. PBMCs) which have established function in antiretroviral transport, researchers may be able to estimate the functional role of the transporters in the immune cells residing in tissues.

2. Further examine the effect of hormones, contraceptives and inflammation, at different concentrations. The concentration dependency will be examined, so that a more comprehensive understanding of the influence of these factors on transporter expression will be obtained.

3. Examine the kinetics of tissue drug concentrations after topical and systemic dosing of the substrate drugs, and acquire tissue drug concentrations at more time points to obtain the AUC with and without the transporter inhibitors. In addition, more doses of the substrate drug and inhibitor need to be tested in future studies to under the effect of substrate and inhibitor concentrations on the feasibility of the inhibitor approach. Finally, the roles of other highly expressed efflux transporters, such as P-gp, Bcrp, in tissue drug exposure remain unknown and should be studied.

4. Test the transporter function in colorectal tract. It has been shown that MRP4 transporter in Caco-2 cells could limit the apical-to-basolateral permeation of acyclovir, which is an MRP4 substrate.[253] In addition to cell-based assays, *in vivo* experiments are also needed to confirm the function of colorectal tissue transporters in the whole tissue exposure of substrate drugs used in PrEP and AIDS treatment.

5. Finally, although the wild type mice is a convenient model to assess the role of transporters in antiretroviral PK, it cannot be infected by HIV-1, and the effect of transporter modulation on antiretroviral efficacy cannot be examined in the wild type mouse model. This limitation can be overcome by using the humanized mice transplanted with functional human lymphocytes. These humanized mice have emerged as powerful tools for the rapid screening of topical and oral PrEP products, and could enable the simultaneous examinations of the effects of transporter modulation on drug PK and PD, in the same model.

5.3 Contribution to the fields of HIV prevention, treatment, and vaginal/colorectal drug delivery

The studies conducted in this dissertation have systematically examined the expression of transporters and nuclear receptors, in humans as well as animal and cell models used in PrEP testing. In addition, these studies have demonstrated the function of the MRP4 transporter in mouse cervicovaginal tissues. The observed effects of menstrual (estrous) cycle, exogenous hormone/contraceptives/cytokines on transporter expression shows that these factors can influence transporter expression, and possibly efflux activity, in mucosal tissues and cells relevant to HIV-1 transmission. The comparisons between human, animal models, and cell lines provides critical information on the validity of application of those models, in microbicide product evaluations. In addition, these studies provide data to inform the rational selection of research models for future study of transporter function. The knowledge generated from the conducted studies has enhanced our understanding of the critical determinants of drug exposure in the tissues and cells important for HIV-1 sexual transmission, and will likely facilitate the

development of novel strategies that could increase the tissue drug exposure and effectiveness of PrEP products.

In addition to the PrEP of HIV-1 sexual transmission, the findings generated from these studies also have implications for the treatment and eradication of HIV-1, in infected patients. Since antiretroviral drug exposure in cervicovaginal and colorectal tissues is inversely correlated with viral shedding in mucosal fluids, the information generated from this dissertation will facilitate the optimization of HAART effectiveness and eradication of HIV-1 particles in these tissue sites, and will in turn contribute to the research efforts toward AIDS cure, and will help reduce the HIV-1 transmission from the receptive sex partners to the insertive partners.

Furthermore, the results from this dissertation also have wide implications on the prevention and treatment of many other diseases, such as reproductive tract cancer and other non-malignant diseases, in which the drug molecules need to reach the female genital tract or colorectal tissue to take effect. Overall, the research reported in this dissertation will facilitate the development and optimization of prevention or treatment strategies for a variety of female health issues and diseases.

APPENDIX

Information of the human tissue donors and tissue usage. *, used in the specified experiment.

Patient #	**Tissue type**	**Age/Age range**	**Gender**	**RT-PCR**	**P-gp staining**	**MRP4 staining**	**BCRP staining**
1	Endocervix	35-40	Female	*	*	*	*
2	Endocervix	20-25	Female	*	*	*	*
3	Endocervix	40-45	Female	*	*	*	*
4	Endocervix	25-30	Female	*			
5	Endocervix	45-50	Female		*	*	*
6	Ectocervix	40-45	Female	*			
7	Ectocervix	45-50	Female	*			
8	Ectocervix	35-40	Female	*			
9	Ectocervix	40-45	Female	*			
10	Ectocervix	45-50	Female	*			
11	Ectocervix	40-45	Female	*			
12	Ectocervix	35-40	Female	*			
13	Ectocervix	40-45	Female	*			
14	Ectocervix	45-50	Female		*	*	*
15	Ectocervix	35-40	Female		*		
16	Ectocervix	35-40	Female		*	*	*
17	Ectocervix	45-50	Female		*	*	
18	Ectocervix	30-35	Female		*	*	
19	Ectocervix	40-45	Female		*	*	
20	Ectocervix	35-40	Female		*	*	
21	Ectocervix	40-45	Female		*		
22	Ectocervix	40-45	Female		*		
5	Ectocervix	45-50	Female		*	*	*
23	Vagina	40-45	Female	*			
24	Vagina	45-50	Female	*			
25	Vagina	45-50	Female	*			
26	Vagina	45-50	Female	*	*	*	*
27	Vagina	45-50	Female	*			
28	Vagina	35-40	Female		*	*	*
29	Vagina	40-45	Female		*	*	*
5	Vagina	45-50	Female		*	*	*
30	Liver	30	Female	*			
31	Liver	35	Female	*			

32	Liver	15	Female	*			
33	Liver	65	Male	*			
34	Liver	49	Female	*			
35	Liver	23	Male	*			
36	Sigmoid colon	70-79	Female	*			
37	Sigmoid colon	70-79	Female	*			
38	Sigmoid colon	40-49	Female	*	*	*	*
39	Sigmoid colon	20-29	Female	*			
40	Sigmoid colon	50-59	Female	*			

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