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Title page

Silencing the breast cancer resistance protein (BCRP) expression and function in Caco-2 cells using lentiviral vector-based shRNA

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Running title page

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A-B, Apical to Basolateral;
B-A, Basolateral to Apical;
BCRP, breast cancer resistance protein;
DAPI, 4', 6-diamidino-2-phenylindole;
DDI, Drug-Drug Interaction;
E3S, estrone-3-sulfate;
FTC, Fumitremorgin C
FITC, fluorescein-5-isothiocyanate;
HBSSg, Hanks' Balanced Salt Solution with glucose
MDR, Multidrug Resistance;
MRP, Multidrug Resistance Protein;
P-gp, P-glycoprotein;
PhA, Pheophorbide A;
qPCR, quantitative PCR;
shRNA, short hairpin RNA;
TEER, transepithelial electrical resistance;

Abstract

A series of stable breast cancer resistance protein (BCRP, ABCG2) knockdown cell lines were produced by transduction of Caco-2 cells with lentiviral vector-based shRNA. Caco-2 cell is a human intestinal-derived cell line widely used to study intestinal drug absorption. Caco-2 expresses three apical drug efflux transporters: BCRP, P-glycoprotein (P-gp; ABCB1) and multidrug resistance protein 2 (MRP2, ABCC2). BCRP and P-gp, in particular, play a significant role in pharmacokinetics due to their expression at several key interfaces. Over-expression of BCRP in cancer cells may also be a mechanism of tumor resistance to chemotherapeutic drugs. The goal of this study was to engineer and characterize Caco-2 cell clones with stable knockdown of BCRP expression. shRNA/BCRP lentiviral particles were used to infect a stable clone of Caco-2 cells. Expression of BCRP was monitored using quantitative PCR (qPCR), western blotting, immunofluorescence microscopy and bidirectional transport of probe substrates, estrone-3-sulfate (E3S) and pheophorbide A (PhA). Based on qPCR, expression of BCRP mRNA was knocked down in five clones with a maximum of 97% silencing in Clone D. Silencing of BCRP gene expression was maintained for at least 25 passages. Expression of BCRP protein was also reduced significantly. Functionally, BCRP knockdown was reflected in significant reduction of the efflux ratio of E3S and PhA. Clone D in particular should be a useful model for identifying and characterizing P-gp substrates and inhibitors without interference from BCRP and/or MRP2. In addition, it can be used in conjunction with wild-type or vector control Caco-2 cells to identify BCRP substrates.

Introduction

The role transporters play in drug-drug interactions is critical because of the profound effect they can have on the way drugs function in the body. Although P-glycoprotein (P-gp) is the best understood efflux transporter, there are other efflux transporters that need to be evaluated. One of these is breast cancer resistance protein (BCRP). BCRP is a member of the ATP-binding cassette (ABC) transporter G family and is also known as ABCG2, ABCP or MXP (Ejendal et al., 2002; Doyle et al., 2003). BCRP is a 655-amino acid polypeptide (72 KD) and contains six putative transmembrane domains and four potential *N* –glycosylation sites (Priebsch et al., 2006). BCRP is similar to half the duplicated P-glycoprotein (P-gp) or multidrug resistance protein 1 (MRP1) molecule and functions as a homodimer bridged by disulfide bonds (Doyle et al., 1998; Kage et al., 2002 and Xia et al., 2005) or, potentially, as a multimer. Like P-gp, BCRP is an efflux transporter that can alter the pharmacokinetics of a co-administered drug, or result in toxicity when inhibited.

BCRP is over-expressed in a variety of human MDR cancer cell lines that exhibit an atypical MDR or a non-P-gp-mediated MDR phenotype. In humans, high BCRP gene expression is linked to chemotherapeutic drug resistance. Elevated expression of BCRP results in resistance of various cancer cell lines to antitumor drugs (Rose et al., 1999). Over expression of BCRP leads to increased drug efflux and reduced intracellular drug concentration, resulting in decreased cytoxicity. Clinically, a correlation exists between BCRP expression and patient outcome in some hematological and solid tumors (Ee et al., 2004).

For drug absorption studies, it is desirable to evaluate the contribution of drug efflux transport proteins to impaired absorption. This can be accomplished by inhibiting the expression or activity of transporters. Low molecular weight pharmacologically active compounds, designated as MDR modulators or chemosensitizers, may circumvent MDR phenotypes by inhibiting efflux pump activities of ABC transporters (Kellen, 2003; Robert et al., 2003; Thomas et al., 2003). However, chemical inhibition of transporters can be problematic because such chemicals could inhibit the function of other cellular proteins, and, thus, confound the results of absorption experiments. Although a few compounds are known to inhibit ABCG2, none of them has been clinically applied for reversal of ABCG2-mediated drug resistance.

Recently, chemically synthesized RNAi molecules have been used to silence expression of various genes in mammalian cell lines. Developments in the use of small interfering RNA (siRNA) to cause specific inhibition of gene expression by triggering the RNA interference (RNAi) pathway, have highlighted the potential use of these types of compounds as therapeutic agents in cancer therapy (Lage, 2005). Inhibition by RNA interference requires shRNA or siRNA sequences that target one or more regions of the membrane efflux transport protein target gene. RNAi has been used to down-regulate the expression of the BCRP gene. Inhibition of BCRP expression by RNAi enhanced the intracellular accumulation of BCRP substrates and restored the sensitivity to cytotoxic compounds transported by BCRP (Priebsch A et al., 2006, Li WT et al., 2005, Ee et al., 2004, Lv H et al., 2007). This gene silencing technique has enormous potential application in the study of drug-drug interactions (DDI), drug absorption and cancer gene therapy.

A major disadvantage of this approach is that RNAi synthesized *in vitro* reduces gene expression only transiently and normal expression is restored a few days after transfection. It is also often limited to cells that are easily transfected. In addition, very little is known about the stability of inhibition of gene expression after several cell passages. Hence, the development of more effective and stable gene nuclear silencing RNA-mediating systems is of interest (Priebsch, 2006). In this study, a lentiviral vector expressing shRNA against BCRP was used to transduce C2BBel cells which are a subclone of Caco-2 cells selected for their more "intestinal cell-like" phenotype (Weinstein et al., 2004). The aim of this study was to take the existing method of BCRP down-regulation using *in vitro* RNAi and to improve upon it by using libraries of synthetic shRNA, and transducing C2BBel cells with viruses carrying expression cassettes encoded with shRNAs against BCRP. These viral vector-based shRNA lentiviral transduction particles for human BCRP were used to knock down the expression of BCRP in C2BBel cells. These transporter-specific knocked down (KD) cells offer advantages over the relatively non-specific pharmacologic inhibitors currently used to evaluate the role of ABCB1, ABCG2 and other efflux transporters in the excretion of drugs and on potential DDI.

Materials and Methods

Chemicals. Digoxin, estrone-3-sulfate (E3S), Tween 20 and all other chemicals were purchased from Sigma (St. Louis, MO). 10 X Tris/Glycine Buffer (TBS) was purchased from BioRad (Hercules, CA), ethanol from Shelton Scientific Inc (Shelton, CT),

fumitremorgin C from Calbiochem (San Diego, CA), pheophorbide A (PhA) from Frontier Scientific (Logan, Utah) and formaldehyde from VWR (West Chester, PA).

Cell lines and Culture. The parental cell line, C2BBe1 (ATCC Accession Number CRL-2102), was used to evaluate gene expression of BCRP and bidirectional transport of drug molecules. Cells were maintained in DMEM containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and streptomycin. Cells were incubated in a humidified atmosphere of 5% CO2 and 90% air. Cell culture media and supplies were obtained from Gibco/Invitrogen (Carlsbad, CA).

shRNA Design and Construction of Anti-ABCG2 shRNA Expression Vectors. The method of RNA interference made use of short hairpin RNAs (shRNA). The advantage of shRNA over siRNA is that the former can lead to long-term inhibition of protein expression. Five Lentiviral Transduction Particles encoding for shRNA against BCRP (shRNA/BCRP) were purchased from Sigma (St. Louis, MO). The lentiviral shRNA vector map is showed in Figure 1. These five 21 nucleotide shRNA duplexes from five different parts of the human BCRP mRNA (Gen Bank Accession No. NM_004827) were designed using the MISSION™ search database at www.sigma-aldrich.com/missionsearch, which is produced and distributed under license from the Massachusetts Institute of Technology. The shRNA sequences tested are presented in Table 1A.

Transduction of shRNA/BCRP into C2BBel cells. C2BBe1, a cell line derived from the Caco-2 cells, which were originally established from a human colon adenocarcinoma, were transduced with lentivirus encoding interfering nucleic acid molecules following the manufacturer's protocol. The cells were plated at a density of 5×10^6 cell/well and

transduced with 1 μg of one of the shRNA/BCRP duplexes in viral vector (MOI =1) in 96 well tissue culture plates. As a control, C2BBel cells were also transduced with the same amount of viral vector containing noncoding shRNA (Sigma, St. Louis, MO). Transduced cells were selected in puromycin-containing (10 μg/ml) cell culture medium. Visible clones were picked from the 96 well plates, expanded in 24 well tissue culture plates, and finally transferred to regular cell culture flasks. All these clones were screened using molecular biology tools. BCRP mRNA expression was determined by RT-PCR amplification and quantitative mRNA expression analysis (qPCR) as described below and protein expression was determined by western blotting using a BCRP specific antibody. For functional characterization of BCRP, bidirectional transport assays were conducted in 12-well Transwell® plates using E3S or PhA as the probe substrate.

Reverse transcription-PCR (RT-PCR) & Quantitative PCR (qPCR). Total RNA was extracted from cells by using TRIzol® reagent (Invitrogen, Carlsbad, CA). Isolation was completed following the manufacturer's protocol. RNA yield and purity were quantified by UV absorbance spectroscopy. RT-PCR was performed using Premium One Step RT-PCR kit (Invitrogen, Carlsbad, CA). Products of RT-PCR were separated and assessed by gel electrophoresis using a 2% agarose gel. For quantitative mRNA expression analysis, the cDNA synthesis reaction was performed using Postscript First Strand cDNA Synthesis Kit (New England Biolabs, Ipswitch, MA). Once cDNA synthesis was verified using standard PCR methods, real-time PCR (qPCR) was performed using a specific primers and probe for each gene with the Light Cycle 480 instrument (Roche Diagnostics, Mannheim, Germany). The cDNA levels of the internal control gene, β-actin were measured and used to normalize the cDNA levels of all genes

in both RT-PCR and qPCR. All the sequences of primers and probes (Table 1B) were designed using Universal Probe Library (Basel, Switzerland) for the qPCR probes. The expression level of mRNA was calculated using the comparative CT method and compared with a calibrator. Accordingly, ΔC_T= (mean BCRP C_T) – (mean β-actin C_T). The $\Delta\Delta C_T$ value is defined as the ΔC_T value of the BCRP in the experimental sample minus the ΔC_T of the control for a calibrator ($\Delta\Delta C_T$ = ΔC_T (BCRP) – ΔC_T (calibrator)). The relative gene expression in a particular sample is then given by the following: relative amount of target = $2^{-\Delta\Delta C}{}_T$ value (Burger et al., 2003).

Western blot analysis for BCRP protein detection. Cell monolayers were grown to confluence in 90% DMEM + 10% FBS. Upon reaching confluence, the medium was removed, and the monolayers were washed with cold PBS and lysed in ice-cold RIPA Lysis Buffer (Santa Cruz BioTech, Santa Cruz, CA). Isolation of protein followed the manufacturer's protocol. The concentration of protein was measured following the protocol of Quant-it Protein Assay kit (Invitrogen, Carlsbad, CA) using the Qubit fluormeter (Invitrogen, Carlsbad, CA). For protein expression analysis, 45 μg of protein were separated on 4-20 % SDS- polyacrylamide gels. Following electrophoresis, proteins were transferred to PVDF membranes using a protocol provided by Bio-Rad (Millipore, Billerica, MA). After the proteins were transferred, the membranes were blocked with 0.2% I-block solution (Applied Biosystems, Foster City, CA.) in Tris-Buffered Saline (TBS) containing 0.05% Tween-20™ (TBST) for 1 hour. Blocked membranes were probed sequentially with anti-human BCRP antibody BXP-21 (Sigma Aldrich, MO), anti-MRP1 antibody IU2H10, anti-MRP2 antibody, anti-P-gp antibody C219 (Abcam, Cambridge, MA), anti-OATP-B antibody P-15 (Santa Cruz, CA) and anti-β-actin (Sigma

Aldrich, MO). After washing, the membranes were reacted with the secondary antibody; goat anti-mouse IgG linked to horseradish peroxidase for BCRP, MRP1, P-gp and β-actin (Zymed, Carlsbad, CA) diluted 1:10,000 for BCRP and β-actin and 1:5,000 for P-gp and MRP1, goat anti-rabbit IgG linked to horseradish peroxidase for MRP2 (Abcam, Cambridge, MA) diluted 1:5,000 and rabbit anti-goat IgG linked to horseradish peroxidase (Zymed, Carlsbad, CA) diluted 1:5,000 for OATP-B. Unbound secondary antibody was removed by washing 3 times with TBST. Protein-antibody complexes were visualized using Super Signal West Femto Chemiluminescent Substrate (Pierce, Rockford, IL) following the manufacturer's protocols.

Immunofluorescence microscopy. For immunocytochemical detection, cells were cultured on D-polylysine pre-coated cover slips (BD Biosciences, Bedford, MA) and grown to high confluence (3-5 days in culture). Cells were fixed using 4% formaldehyde diluted in PBS for 10 min at room temperature. Nonspecific binding sites were blocked by incubation in PBS containing 3% BSA for 30 min after washing. Cells were incubated with anti-human BCRP Ab conjugated with FITC (clone 5D3, Santa Cruz, CA) at 4^0C overnight. The cellular nuclei were stained using 0.36 mM DAPI (1:500 from 180 mM stock, Invitrogen, Eugene, OR) in PBS for 10 min at room temperature. After being washed cover slips were mounted onto slides in Vectashield solution (Vector Labs, Burlingame, CA). Cover slips on slides were stabilized with nail polish and dried at room temperature for 10 min. Visualization of protein expression and cellular localization was done using a Leica TCS laser scanning confocal microscope.

Bidirectional Permeability Studies. The passive permeability of compounds through cells monolayers correlates well with *in vivo* absorption in humans (Artursson et al.,

2001). In this study, permeability assays were performed as previously described (Wang et al., 2005). Both the BCRP knockdown and control cells were grown on Transwell inserts (12-well Costar Transwell®) to form monolayers under conditions normally employed for growing C2BBel cells culture (21 days after seeding). The BCRP substrates, E3S (Xia et al., 2005) or PhA (Robey et al., 2004) were used in bidirectional permeability assays to determine BCRP function. On pre-determined days post-seeding, monolayer integrity was monitored using transepithelial electrical resistance (TEER). Additionally, bidirectional transport experiments were conducted using a solution containing 10 μM propranolol, 10 μM atenolol, 10 μM digoxin, and 5 μM E3S. The total duration of the transport experiments was two hours. Measurements of the concentration of compounds in the donor and receiver buffers were performed by LS/MS/MS. PhA was prepared at 10 μM in HBSSg buffer containing 1% bovine serum albumin (BSA). Bidirectional transport experiments for PhA were performed using the same protocol as that for E3S on Transwells® plated with vector control cells or Clone D cells. For apical-to-basolateral (A-to-B) transport, 0.5 mL of Ph A solution was dosed to the top chamber (insert) and 1.5 mL of blank HBSSg buffer containing 1% BSA was applied to the bottom chamber (well). For basolateral-to-apical (B-to-A) transport, 1.5 mL of PhA solution was dosed to the bottom chamber (well) and 0.5 mL of blank HBSSg buffer containing 1% BSA was added to the top chamber (insert). At 60 and 120 min, 100 μL aliquots were sampled from the receiver chambers. The concentration of PhA was measured using a BMG microplate reader (Fluostar 403, BMG Laboratory, Offenburg, Germany) with excitation and emission wavelengths at 390 nm and 680 nm, respectively.

The BCRP inhibitor, fumitremorgin C (FTC) was used to block the residual BCRP transport activity remaining in the shRNA/BCRP clone D cell line in order to compare this reduced activity to BCRP activity in vector control cells. Cell monolayers in Transwells® were preincubated with either 10 μM FTC or with blank HBSSg buffer for 30 minutes. After preincubation, the solutions were aspirated from the donor side and replaced with fresh HBSSg solution containing 10 μM E3S in the presence or absence of 10 μM FTC. Samples were collected at 0 and 120 minutes from the donor chambers and at 60 and 120 minutes from the receiver chambers. Concentrations of E3S in the samples were determined by LC-MS/MS.

The apparent permeability coefficient (Papp) was calculated as previously reported (Wang et al., 2005) and efflux ratios, i.e. basolateral to apical (Papp B → A) /apical to basolateral (Papp A → B), were used as a measure of functionality. The efflux ratios of E3S or PhA in shRNA/BCRP knockdown clones were compared to that in the non-coding shRNA/vector control transduced cells to evaluate functional changes in efflux due to BCRP knockdown. The permeability of Lucifer yellow, a monolayer integrity marker compound, was also measured for each monolayer using fluorescence detection. Lucifer yellow Papp values were calculated to determine whether monolayer integrity was impaired during the permeation study. Monolayers which exhibited abnormally high Lucifer yellow permeability values were excluded from further analysis.

Statistical Analysis. Student's *t*-test was used to evaluate statistical significance. A *P*-value < 0.05 was set as the criteria for statistical significance.

Results

Suppression of BCRP mRNA and protein expression by shRNA/BCRP containing lentiviruses. Five different shRNA constructs targeting BCRP mRNA (shRNA/BCRP lentiviruses A to E) were transduced into human C2BBel cells to inhibit the expression of the BCRP-encoding mRNA. BCRP mRNA expression was analyzed using RT-PCR and real-time qPCR. BCRP protein expression was determined by western blotting. All five shRNA/BCRP constructs caused a significant reduction of BCRP mRNA expression relative to the mRNA level in the non-coding shRNA vector control C2BBe1 cells (VC) as shown by RT-PCR (Figure 2) and qPCR (Figure 3). The relative expression levels of BCRP mRNA ($2^{-\Delta\Delta C}{}_T$) in shRNA/BCRP clone A to E knockdown cells (which ranged from 0.03 to 0.47) were significantly lower than in vector control transduced cells (1.02), as shown in Figure 3. Clones C and D knockdown cells showed the highest knockdown. These results indicate that although all five shRNA/BCRP inhibit production of BCRP mRNA in C2BBel cells, two of the constructs were (C and D) more potent inhibitors of BCRP mRNA expression.

The inhibition of protein expression by transduction with the shRNA/BCRP-containing lentivirus was confirmed by western blot analysis (Figure 4). All five shRNA/BCRP lentivirus transduced cell lines produced much less BCRP protein than the vector control transduced cells. The rank order of BCRP expression levels, assayed by RT-PCR, qPCR and western blot was similar for the five shRNA constructs, indicating that different shRNA sequences have different efficiencies for suppressing BCRP activity in C2BBe1 cells.

The stability of the inhibition of expression of BCRP mRNA was examined in shRNA/BCRP clone D knockdown cells by qPCR. The results indicate that down-

regulation of expression of BCRP mRNA is constant from cell passage 10 through 25 (Figure 5).

Effect of BCRP knockdown on expression of other transporters in shRNA/BCRP knockdown cells. Since expression of other efflux transporters, such as MRP1, MRP2, P-gp and of uptake transporters, such as OATP2B1 has been reported in C2BBel cells, we evaluated the effect of BCRP knockdown on expression of these transporters in the shRNA/BCRP knockdown cells to confirm that the knockdown effect was specific for BCRP. Figure 2 and 4 present expression of mRNA and protein in shRNA/BCRP knockdown cells. There were no decreases in transporter gene expression in the knockdown cells compared to control vector cells. These results indicate that the knockdown of BCRP expression was specific and that expression of other transporters was not decreased by BCRP knockdown.

Expression of human BCRP protein in individual C2BBel cells, vector control cells and shRNA/BCRP clone D knockdown cells. Both mRNA and protein levels provide a measure of the average knockdown, but they do not indicate how the protein is distributed in a population of BCRP knockdown cells. We therefore examined the expression and distribution of the BCRP protein in knockdown cells by using laser scanning confocal microscope for immunocytochemical detection (Figure 7). There was a strong overall decrease of the BCRP immunofluorescence signal in clone D knockdown cells compared to wild type C2BBel cells and vector control transduced cells.

Characterization of the cell monolayer barrier properties and function of BCRP in shRNA/BCRP lentivirus-transduced C2BBel cells. Bidirectional transport of E3S was used to characterize the function of BCRP in shRNA/BCRP knockdown cells. Results

(Table 2) indicate that the E3S efflux ratio was significantly reduced compared to vector control cells, indicating that BCRP activity was inhibited in shRNA/BCRP knockdown cells. Clone D showed the highest inhibition of BCRP function as evidenced by 90 % decrease in the efflux ratio of E3S. Atenolol and propranolol (passive diffusion markers) exhibited A to B Papp values similar to those obtained in vector control cells, implying that BCRP knockdown did not affect either the passive transcellular or passive paracellular diffusion pathways. The transport results also showed that transport of E3S in the B to A direction was significantly reduced in the BCRP knockdown cells compared to vector control cells (Figure 6). In contrast, the A to B transport of E3S was not appreciably changed in BCRP knockdown cells. To evaluate the specificity of inhibition for BCRP transport activity in shRNA/BCRP clone cells, we also conducted efflux study by using another BCRP substrate (PhA), which has been reported to be a more specific substrate for BCRP (Robey et al, 2004). The results indicate that the transport of PhA was significantly inhibited in shRNA/BCRP clone D knockdown cells and that the inhibition was greater than that observed with E3S efflux (Table 3)

To further examine the reduction of BCRP efflux in shRNA/BCRP clone D knockdown cells, bidirectional transport experiments were performed at 10, 15, 21 and 25 days post-seeding. Results are listed in Table 4 A and B. All cell monolayers developed TEER > 400 $\Omega \cdot cm^2$. The passive diffusion marker compounds, atenolol and propranolol, showed distinct low and high permeability coefficients, indicating normal monolayer discrimination (results not shown). The vector control cells exhibited high efflux of E3S (efflux ratio >18), whereas the Clone-D cells showed a low E3S efflux (efflux ratio < 2). FTC treatment of Clone D knockdown cells resulted in a further decrease in the E3S

efflux ratio, suggesting that the residual E3S efflux seen in the knockdown cells was associated with residual BCRP activity. The residual BCRP transport activity was 8.86 % compared to C2BBel wild type cells (Table 5).

The phase contrast pictures of the cultured cells showed no apparent morphological differences between knockdown cells and control cells (data was not shown). Results indicate that BCRP knockdown using lentiviral shRNA particles did not result in discernable morphological changes.

Discussion

The mechanism of RNAi-triggered mRNA destruction is widely used as a powerful tool for modulation of gene expression. This technique has been under extensive investigation and has been applied recently to the inhibition of MDR1 (Stege et al., 2004; YagÜe et al., 2004) and BCRP expression (Ee et al., 2004, Li et al., 2005, Priebsch et al., 2006 and Lv et al., 2007). Results from these studies have shown that RNAi is useful for down regulation of BCRP. However, a major disadvantage of this approach is the transient duration of the suppression of gene expression caused by chemically synthesized siRNAs following the transfection with RNAi constructs. Hence, the development of more effective and stable gene-silencing RNAi-mediated systems is desirable.

In this study, we used lentiviral vector-based shRNA/BCRP to establish a C2BBel-derived cell lines with stable suppression of BCRP gene expression. As a first step, we confirmed that transduction by all five shRNA/BCRP lentiviral vector constructs produced a pronounced BCRP gene-silencing at the mRNA and protein levels. The

knockdown efficiency of BCRP mRNA expression in the five shRNA/BCRP transduced cell lines ranged from 54 to 97 %. It is known that not all positive synthetic shRNA oligos work in vector-based RNAi (Celius et al., 2004). The silencing may depend on the efficiency of transduction and the half-life of the shRNA/BCRP. It is also not clear the accuracy with which the efficiency of an individual siRNA can be predicted (Schuck et al., 2004). Our results also confirm that following the introduction of shRNA into cells not all shRNA are capable of knocking down BCRP mRNA expression to the same extent. The different proportion of mRNA that was knocked down by the five shRNAs suggests that each shRNA insert differed in its ability to mediate RNAi knockdown (Brummelkamp et al., 2002). Therefore, subsequently, we investigated the characterization of one transduced shRNA/BCRP clone (clone D) of the five shRNA/BCRP knockdown clone cells we created. Clone D had the highest knockdown of mRNA expression (97 % down-regulation compared to vector control cells) and the highest reduction of activity as indicated by more than 87 % reduction of the efflux ratio of the BCRP substrates, E3S and PhA, compared to vector control transduced cells. Furthermore, the inhibition of E3S transport in clone D cells using FTC indicated that the residual BCRP transport activity was less than 10 % compared to C2BBel wild type cells.

It has been reported that PhA is transported selectively by BCRP and not by P-gp or other ABC transporters (Robey et al, 2004). Our results support this conclusion, because the compound was not effluxed in the knockdown cells even though these cells continued to express MDR1 (human P-gp) and MRP2. Examination of the expression of other efflux and uptake transporters (MRP1, MRP2, OATP2B1 and P-gp) indicated that such expression is unaffected by BCRP knockdown in C2BBel cells, suggested the inhibition

of BCRP gene expression and transport activity in shRNA/BCRP knockdown cells was selective and specific for BCRP.

The clone D knockdown cell line established in the present study is stable in culture for at least 25 cell passages based on functionality and BCRP levels. This duration is much longer than the effect observed after transduction with either chemically synthesized transient siRNA or non viral vector-based siRNA. Both of these of strategies are limited by low transfection efficiency and short-term cellular persistence of siRNA molecules (Nieth et al., 2003; Wu et al., 2003). With a long duration of the knock down phenotype, it is possible to perform studies over an extended time frame without the need for frequent transfections. Additionally, one may circumvent potential increases in phenotypic variability and repeated characterization of the new clones, which would be required for each additional transfection. More stable knockdown cells also confer numerous economical and temporal advantages.

Immunocytochemical detection revealed that BCRP, which is constitutively expressed on C2BBel cells, is significantly reduced in BCRP knockdown clone D cells (Figure 7). Our results correspond with an earlier report, that found BCRP is localized predominately on the apical membrane (Matsushima et al., 2005).

In the present work, the effect of BCRP/shRNA on the functional expression of BCRP was determined by measuring bidirectional E3S and PhA permeability through C2BBel control vector-transduced or BCRP/shRNA vector transduced cell monolayers. The efflux ratio, calculated to evaluate the BCRP phenotype, showed that transduction using shRNA/BCRP lentiviral vectors effectively down-regulated endogenous BCRP efflux activity. The decreases in mRNA and protein levels closely corresponded with the

changes in the BCRP efflux phenotype of the knockdown clones compared to C2BBel cells. The knockdown clones exhibited a significant decrease (44 to 90 % compared to vector control cells, Table 2) in the efflux of E3S. Also, the decrease in the efflux of E3S across shRNA/BCRP clone D knockdown cell monolayers was stable from days 10 through 25 in culture. The stability of the E3S efflux ratio indicates that this function is relatively insensitive to monolayer tightness because TEER values were lower between days 10 to 15 than after the monolayers reached full development (≥ 21 days). After 21 days in culture, TEER values of clone D knockdown cell monolayers were within the normal range expected for the parental cell line C2BBe1, as were the permeability values for atenolol and propranolol and the digoxin efflux ratios. These results indicate that the time suitable to perform permeability studies in clone D cells is 21-25 days, which also allows for comparable barrier properties and functional P-gp activity between the clone D cell line and the parental cell line. The cell morphology is similar in BCRP clone D knockdown cells compared to the parental cell line (data was not shown). Therefore, the stable knockdown cells developed here should provide a way of evaluating the importance of the role of BCRP in drug absorption and transport by comparing drug efflux from the knockdown and parental or vector control cell lines.

In summary, our study has demonstrated stable silencing of the expression of BCRP gene and the accompanying decrease in BCRP transport function in human C2BBel cells can be accomplished by using viral vector based shRNA. These transporter-specific knockdown cells used alone or together with the relatively non-specific pharmacologic inhibitors, may provide a novel and effective laboratory tool to evaluate the role of BCRP in drug and nutrient efflux and drug-drug interactions.

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Footnotes.

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Legends for figures

Figure 1. Physical map of lentiviral shRNA transfer vector constructs (from Sigma). BCRP sequences are cloned into pLKO.1-puro vector. In this vector, U6 is a robust pol III promoter with precise initiation and termination of shRNA transcription.

Figure 2. Effects of BCRP knockdown on expression of BCRP, MRP1, MRP2, OATP2B1 and P-gp mRNA in shRNA/BCRP knockdown cells. 2 μg of RNA from each batch of cells was used for RT-PCR and the products of RT-PCR analysis using 2 % agarose gel. VC: shRNA vector control transduced cells. A to E: shRNA/BCRP knockdown cell clones.

Figure 3. Relative mRNA expression levels of BCRP in knockdown cells, as estimated by quantitative real-time PCR (qPCR). The level of mRNA expression in the parental C2BBe1 cell line was set = 1.0 for reference purposes. VC: shRNA vector control cells; A to E: shRNA/BCRP clones cells. 10 ng of cDNA synthesized from total RNA were used for qPCR. β-actin was used as a reference gene to calculate $^{\Delta C}{}_{T}$ and the results expressed as $2^{-\Delta\Delta C}{}_{T}$ for all five shRNA/BCRP clone cells. Each column and vertical bar represents the mean ± S.D. of three determinations. *, $P < 0.05$ and **, $P < 0.01$ statistically different from vector control cells.

Figure 4. Expression of BCRP, MRP1, MRP2, OATP2B1 and P-gp protein in shRNA/BCRP knockdown cells. 45 µg protein was used for western blot assay. A to E: shRNA/BCRP knockdown cell clones. VC: shRNA vector control tranduces cells.

Figure 5. Expression of BCRP mRNA in shRNA/BCRP clone D knockdown cells between cell passages 10 and 25 after transduction. VC: shRNA vector control cells (passage# 9 after transduction). The number above the bar is the relative level of BCRP mRNA expression compared to the parental C2BBe1 cells.

Figure 6. Time profiles for the bidirectional transport of E3S across BCRP clone D knockdown cells and vector control cells monolayers. Each point and vertical bar represents the mean ± S.D. of three determinations. Where vertical bar are not shown, the S.D was contained within the limits of the symbol.

Figure 7. Laser scanning confocal microscopic analysis of the immunocytochemical distribution of human BCRP protein (FITC, green) and of cellular nuclei (DAPI, blue) in knockdown cells and control cells. BCRP protein and cellular nuclei were visualized by immunofluorescence (objective 60 X). A, C2BBe1 cells; B, shRNA vector control transduced cells; C, BCRP clone D knockdown cells.

Tables (total 5)

TABLE 1

Sequences of shRNA/BCRP clones (table 1A) and of primers and probes (table 1B)

Table 1A. Sequences of shRNA/BCRP

shRNA/BCRP clone	Sequence
A	CCGG***GCCTCGATATTCCATCTTCAA*** CTCGAG***TTGAAGATGGAATATCGAGGC*** TTTTTG
B	CCGG***GCAACAACTATGACGAATCAT*** CTCGAG***ATGATTCGTCATAGTTGTTGC*** TTTTTG
C	CCGG***CCTTCTTCGTTATGATGTTTA*** CTCGAG***TAAACATCATAACGAAGAAGG*** TTTTTG
D	CCGG***GCTGTGGCATTAAACAGAGAA*** CTCGAG***TTCTCTGTTTAATGCCACAGC*** TTTTTG
E	CCGG***CCTGCCAATTTCAAATGTAAT*** CTCGAG***ATTACATTTGAAATTGGCAGG*** TTTTTG

Table 1B. Sequences of primers and probes

BCRP	
Forward primer	TGGCTTAGACTCAAGCACAGC
Reverse primer	TGGTCCCTGCTTAGACATCC
Probe	TGCTGTCC
MRP1	
Forward primer	TGTGGGAAAACACATCTTTGA
Reverse primer	CTGTGCGTGACCAAGATCC
Probe	GGATGCTG
MRP2	
Forward primer	AGTGAATGACATCTTCACGTTTG
Reverse primer	CTTGCAAAGGAGATCAGCAA
Probe	TCCTCAGC
OATP2B1	
Forward primer	GGCGAAAGGTCTTAGCAGTC
Reverse primer	GCTTAGAGGGAGAGTCCTTGC
Probe	GCCAGGAA
P-gp	
Forward primer	ACAGAAAGCGAAGCAGTG GT
Reverse primer	ATGGTGGTCCGACCT TTTC
Probe	TTGCTCTG
β-actin	
Forward primer	ATTGGCAATGAGCGGTTC
Reverse primer	GGATGCCACAGGACTCCAT
Probe	CTTCCAGC

TABLE 2

Mean Papp values (x 10^{-6}cm/s) and Efflux Ratio for E3S tested in five shRNA/BCRP knockdown clones (A to E) and in shRNA vector control transduced cells.

	Control	Clone A	Clone B	Clone C	Clone D	Clone E
E3S, Efflux ratio (Papp(B-A)/(A-B))	25.58	14.42	10.23	3.28	2.61	5.70
Atenolol (Papp, A-B)	0.13	0.25	0.12	0.13	0.41	0.09
Propranolol (Papp, A-B)	17.23	22.44	19.88	19.97	23.23	21.12

TABLE 3

The bidirectional transport of pheophorbide A in BCRP clone D knockdown and vector control transduced cells. All results were from triplicate samples.

Cell line	Papp ($\times 10^{-6}$cm/s)		Efflux ratio	Inhibition (%)
	A-B	B-A	(B-A/A-B)	compared to VC
Vector control (VC)	12.49 ± 8.24	39.33 ± 11.01	3.15	
shRNA/BCRP Clone D	22.81 ± 3.43	21.42 ± 5.28	0.94	~100

TABLE 4

Summary of TEER, Papp values (x 10^{-6}cm/s) and Efflux Ratios for E3S in shRNA/BCRP clone D knockdown cells (table 4A) and in shRNA vector control transduced cells (table 4B). All results were from triplicate samples.

Table 4A

Days in culture	10	15	21	25
TEER (ohm · cm2, n=6)	181	293	771	683
Papp, E3S A to B	5.16	4.15	2.97	2.06
Papp, E3S B to A	6.04	6.76	4.63	3.3
Efflux ratio (Papp(B-A/A-B))	1.17	1.63	1.56	1.6

Table 4B

Days in culture	10	15	21	25
TEER (ohm · cm2, n=6)	269	386	513	507
Papp, E3S A to B	0.81	0.69	0.55	0.44
Papp, E3S B to A	15.1	16.94	15.57	10.78
Efflux ratio (Papp(B-A/A-B))	18.65	24.59	28.39	24.59

TABLE 5

Residual of BCRP activity in presence and absent of 10 μM fumitremorgin C (FTC) in BCRP clone D knockdown cells. The results of Papp values and efflux ratios for E3S were from triplicate samples. The residual of BCRP activity in clone D knockdown cells or FTC treated cells was calculated by dividing by the value of efflux ratio across Caco-2 wt cells.

Cell line	Papp (×10-6cm/s)		Efflux ratio	Residual of BCRP
	A-B	B-A	(B-A/A-B)	activity in function (%)
Caco-2 wt	0.94 ± 0.23	20.24 ± 2.58	21.45	100
Caco-2 wt + FTC	2.45 ± 0.49	1.10 ± 0.28	0.45	-2.70
Clone D	1.33 ± 0.07	3.66 ± 0.45	2.75	8.56
Clone D +FTC	1.21 ± 0.26	0.60 ± 0.10	0.49	-2.48

Figure 1

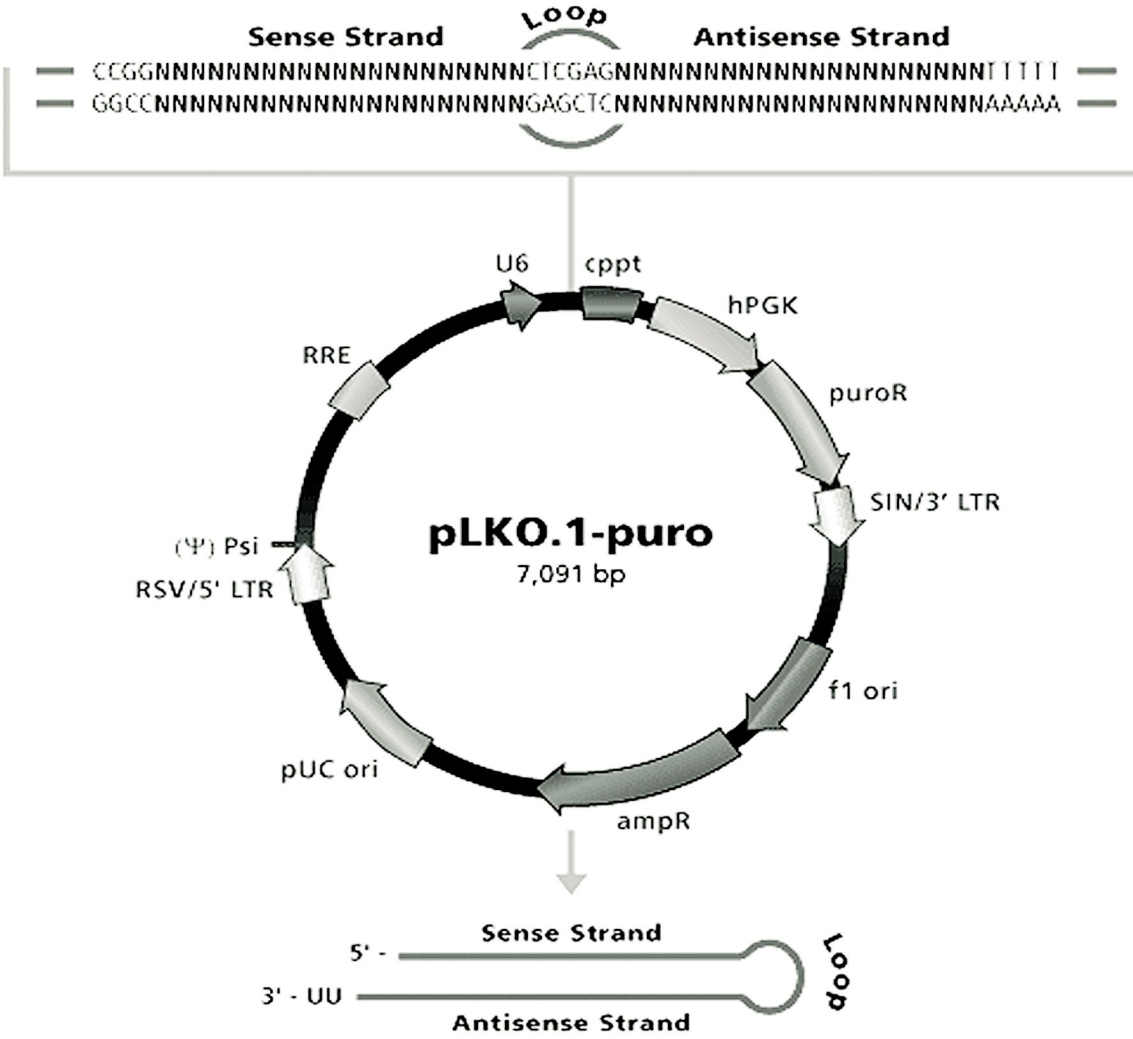
Sense Strand
Loop
Antisense Strand
CCGGNNNNNNNNNNNNNNNNNNNCTCGAGNNNNNNNNNNNNNNNNNNNTTTTT
GGCCNNNNNNNNNNNNNNNNNNNGAGCTCNNNNNNNNNNNNNNNNNNNAAAAA
U6
cppt
hPGK
puroR
SIN/3' LTR
f1 ori
ampR
pUC ori
RSV/5' LTR
(Ψ) Psi
RRE
pLKO.1-puro
7,091 bp
5' -
Sense Strand
3' - UU
Antisense Strand
Loop

Figure 2

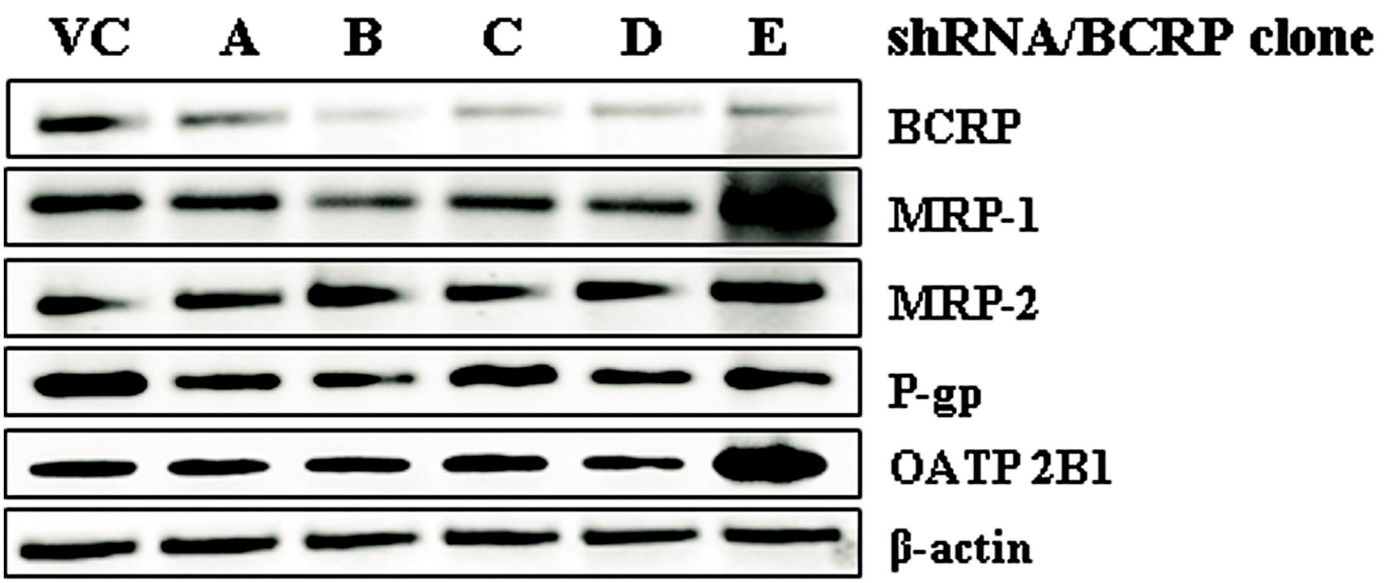

Figure 3

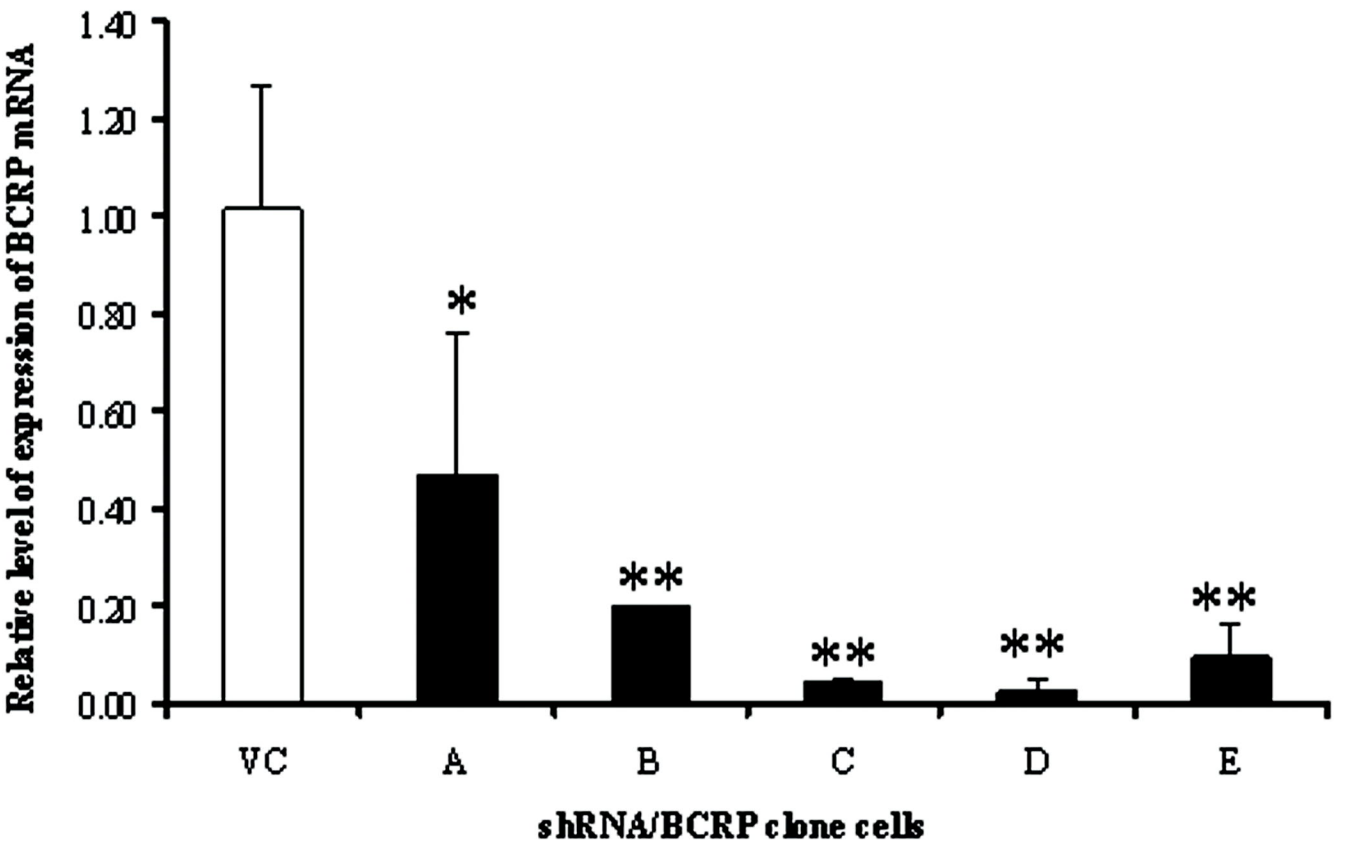
Relative level of expression of BCRP mRNA
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
*
**
**
**
**
VC
A
B
C
D
E
shRNA/BCRP clone cells

Figure 4

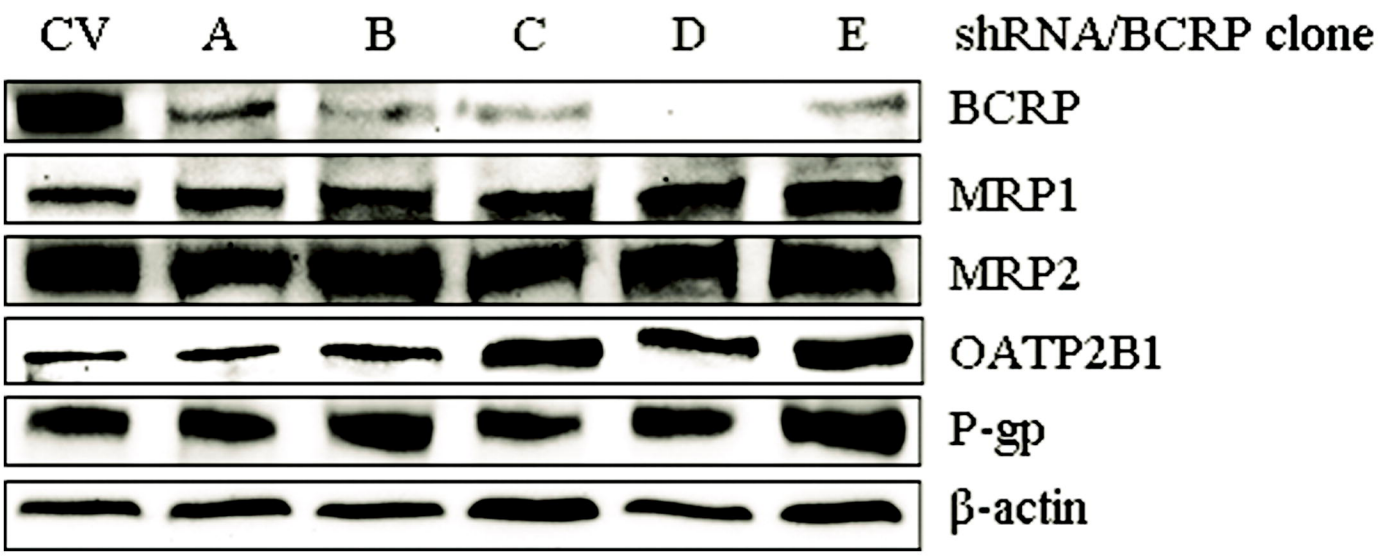

Figure 5

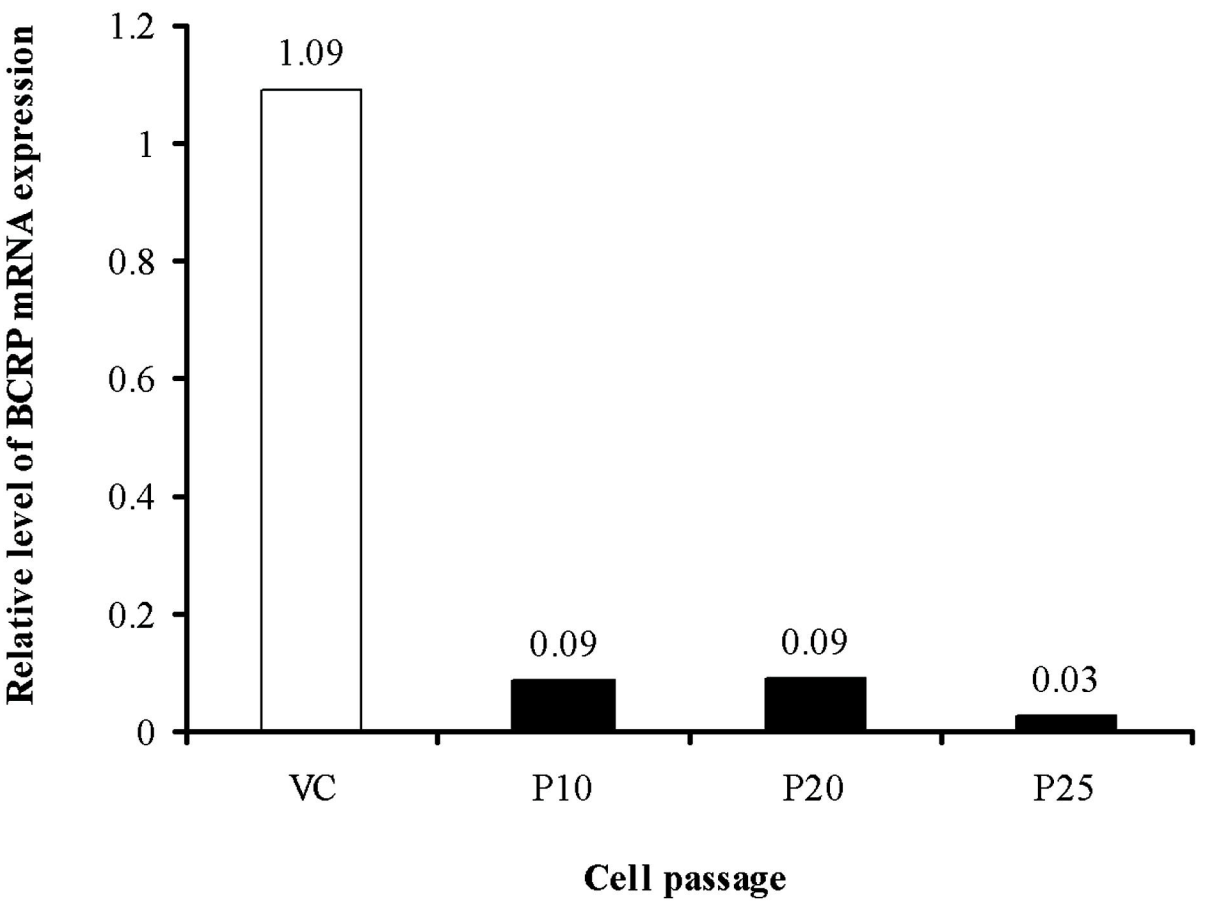
Relative level of BCRP mRNA expression
1.2
1
0.8
0.6
0.4
0.2
0
1.09
0.09
0.09
0.03
VC
P10
P20
P25
Cell passage

Figure 6

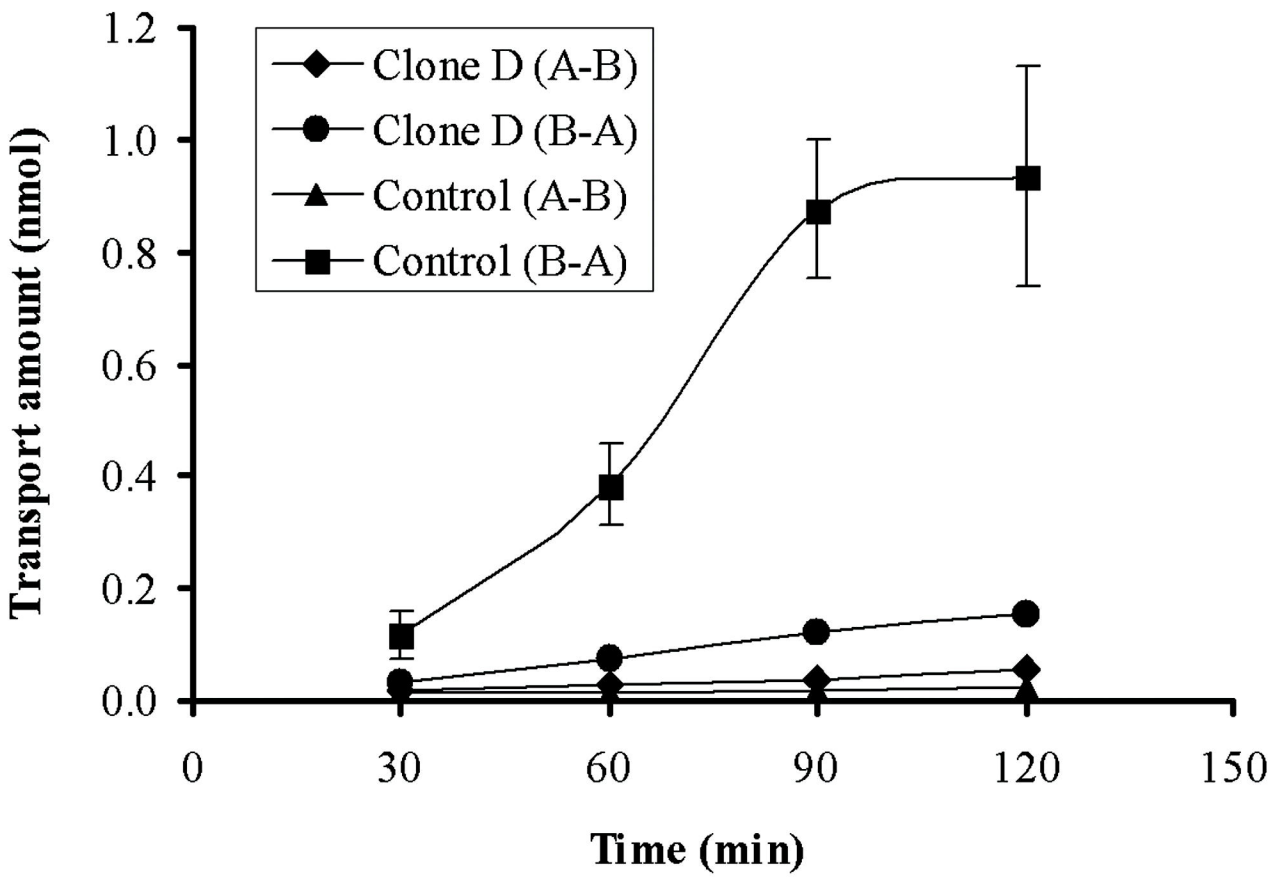
Clone D (A-B)
Clone D (B-A)
Control (A-B)
Control (B-A)
Transport amount (nmol)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
30
60
90
120
150
Time (min)

Figure 7