A Model of Secreting Murine Mammary Epithelial HC11 Cells Comprising Endogenous Bcrp/Abcg2 Expression and Function

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Abstract

Breast cancer resistance protein (Bcrp/Abcg2) and multidrug transporter 1 (Mdr1/Abcb1) are efflux proteins located in the apical membrane of mammary epithelial cells (MEC). Bcrp is induced in MEC during gestation and lactation, while Mdr1 is down-regulated during lactation. Numerous drugs and toxic compounds are known to be actively secreted into milk by Bcrp, but most chemicals have not been investigated in this respect, emphasizing the need for functional Bcrp studies in an established cell line with secreting mammary epithelial cells. The present study was undertaken to examine expressions of Bcrp and Mdr1 in mammary epithelial HC11 cells, derived from a mid-gestational murine mammary gland. In addition, Bcrp function was assessed by transport experiments with mitoxantrone (MX) in undifferentiated HC11 cells, in HC11 cells subjected to *Bcrp* RNA interference (RNAi) as well as in HC11 cells stimulated to differentiate by treatment with lactogenic hormones. Differentiated HC11 cells organized into alveolar-resembling structures and gene expression of the major milk protein β-casein was induced, whereas undifferentiated cells formed monolayers with lower β-casein expression. Bcrp and Mdr1 gene and protein were expressed in both undifferentiated and differentiated HC11 cells. Differentiation of HC11 cells resulted in increased Bcrp protein expression, while Mdr1 gene and protein expressions were reduced. The Bcrp inhibitor elacridar (GF120918) reduced secretion and increased accumulation of MX in both undifferentiated and differentiated HC11 cells. Silencing of the *Bcrp* gene caused an increased accumulation of MX. The results indicate that the HC11 cell model provides a promising tool to investigate transport of potential Bcrp substrates in mammary epithelial cells.

Introduction

Breast cancer resistance protein (BCRP/ABCG2) is a 72 kDa protein, composed by a single N-terminal ATP binding site followed by six putative transmembrane segments, belonging to the ATP binding cassette (ABC) superfamily of transporters (Doyle et al., 1998). BCRP is situated in the apical membrane of epithelial cells in numerous tissues including the intestine, liver, kidneys and several blood-tissue barriers where it serves to extrude its substrates and by these means limiting availability and systemic exposure (Jonker et al., 2000; Maliepaard et al., 2001; Zhang et al., 2004). Furthermore, BCRP is highly expressed in cancer cells where it prevents the uptake of the antineoplastic drug mitoxantrone, diminishing its therapeutic effect (Doyle et al., 1998; Bates et al., 2001). Thus, one important biological function of BCRP in most tissues appears to comprise cellular protection and detoxification.

BCRP is also expressed in apical membrane of mammary epithelial cells (MEC) which faces the alveolar lumen of the mammary glands (Jonker et al., 2005; Lindner et al., 2013). During gestation and lactation BCRP is upregulated. Although the physiological role for BCRP in MEC is not totally clarified the transporter has been demonstrated to actively efflux vitamin B2 (riboflavin) into milk (van Herwaarden et al., 2007; Vlaming et al., 2009). Furthermore, active secretion of a variety of exogenous BCRP substrates, including human and veterinary drugs, environmental pollutants and other food contaminants, into milk have been demonstrated (Jonker et al., 2005; van Herwaarden et al., 2007; Mealey 2012). Expression and function of BCRP can also be influenced by chemicals. Thus, flavonoids and isoflavonoids, present in many food and feed plants, inhibit BCRP and may thus decrease milk secretion of BCRP substrates (Zhang et al., 2004; Morris and Zhang, 2006). On the other hand, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and the fungicide prochloraz induce

BCRP in mammary epithelial cells and may increase the milk excretion of BCRP substrates (Halwachs et al., 2013).

The exposure of hazardous compounds via milk in sensitive population groups is of high concern in risk assessment and there is a need for methods to screen for chemicals with active transport into milk and to further investigate the underlying mechanisms. Transfected Madin-Darby Canine Kidney II (MDCKII) epithelial cells stably expressing human, bovine and caprine BCRP have been used to identify new BCRP substrates as well as the impact of the common Y581S point mutation in BCRP function (Jonker et al, 2000; Real et al., 2011; Wassermann et al., 2013; Halwachs et al., 2014). However, expression and function of endogenous BCRP in non-tumorigenic mammary epithelial cell lines has not yet been examined. Such a model would be valuable for studies on BCRP expression and function after treatment with xenobiotics.

HC11 cells are non-tumorigenic mammary epithelial cells derived from the mouse mammary epithelial cell line COMMA-1D collected from the mammary glands of Balb/c mice during mid-gestation (Danielson et al, 1984). The HC11 cells express functional prolactin receptors and have been used as a model to examine the progression of mammary epithelial differentiation to a secreting phenotype (Ball et al., 1988; Marte et al., 1994; Desrivières et al., 2003). In addition, the HC11 cell model has been applied to characterize transporters implicated in the flux of zinc, copper, sodium and calcium across the membranes of the lactating mammary epithelium (Kelleher and Lönnerdal, 2005, 2006; Boyd and Náray-Fejes-Tóth, 2007; Öhrvik et al, 2010; McCormick and Kelleher, 2012; Ross et al., 2013). However, no studies have so far been directed to investigating expression and function of ABC transporters in HC11 cells.

The present study was undertaken to examine endogenous expressions of Bcrp and Mdr1 in mammary epithelial HC11 cells. In addition, transport experiments with the BCRP substrate mitoxantrone (MX) were carried out in undifferentiated HC11 cells, in HC11 cells subjected to *Bcrp* RNAi as well as in HC11 cells stimulated to differentiate by treatment with lactogenic hormones.

Materials and Methods

Cells

HC11 cells of passage 26 was a generous gift from Dr Shannon Kelleher, Department of Nutritional Sciences, Pennsylvania State University, USA and was cultured by permission of Dr Bernd Groner, Institute for Biomedical Research, Frankfurt, Germany. The cells were expanded in T75 tissue culture flasks at 37°C in 15 ml of sterile filtered RPMI 1640 medium containing L-glutamin and 25 mM HEPES (Invitrogen) supplemented with 50 μg/ml gentamicin, 5 μg/ml bovine insulin, 10 ng/ml epidermal growth factor (EGF), 7.5% NaHCO3 and 10% heat-inactivated foetal bovine serum (FBS) in an atmosphere of 95% air and 5% CO2 in 95% relative humidity. The cell culture medium was changed every 2-3 days and cells between passages 28-33 were harvested at 80-90% confluency. HC11 cells were seeded at a density of 50,000 cells/cm^2 in 6-well plates and cultured to confluency. Two days postconfluency cells were cultured for an additional 24 h in the FBS-containing RPMI 1640 medium described above but without EGF. Differentiation of the HC11 cells was then achieved by culturing the cells for 72 h in EGF- and FBS-free RPMI 1640 medium supplemented with1 μg/ml prolactin and 1 μM cortisol as described (Desrivières et al., 2003). Undifferentiated HC11 cells (controls) were cultured in parallel with the FBS- and EGF-containing RPMI 1640 medium. To study the cellular organization approximately 800,000 cells in a volume of 400 μl were seeded per chamber on slides (Lab-Tec® chamber slide™ system, Nalge Nunc International). In 4 of the 8 chambers on each slide (n=3) the cells were allowed to differentiate for 3 days as described above whereas the other 4 served as undifferentiated controls. The cells were fixed in 4°C acetone for 5 min and then rinsed with 1XPBS, pH 7.4 prior to nuclei staining with hematoxylin.

Quantitative real-time RT-PCR

Following incubation of the HC11 cells in either control or differentiating culture medium as described above or transfection medium as described below the cells were rinsed with 1XPBS and the RNA was isolated by applying NucleoSpin®RNA II Columns with DNaseI according to the instructions of the manufacturer (BD Biosciences). As a quality control of primers used in the real-time RT-PCR with cellular RNA, primers were tested on RNA from mouse mammary gland tissues taken at different lactation stages. HARLAN NMRI-mice were given a standard pellet diet and tap water ad libitum under standard conditions of temperature and light. Animals were killed by cervical dislocation and mammary glands from pregnant (gestation days 13 and 18), lactating (lactation days 2 and 9) and weaning (weaning day 2) mice were rapidly excised, placed in RNAlater (Invitrogen), snap-frozen in liquid nitrogen and stored at -70°C pending isolation of total RNA. Tissues were homogenized in 10 volumes of RA1 buffer as recommended by the manufacturer (BD Biosciences) and RNA isolated with NucleoSpin®RNA II Columns with DNaseI as described above. Animal experiments were ethically approved (permit no. 2012-15-2934-00587) and carried out in collaboration with Dr Christopher Knight, at the Department of Health and Medical Sciences, University of Copenhagen, Denmark. Total RNA from mammary glands was quantitated by use of the RiboGreen protocol with DNase I (Invitrogen) and RNA samples were stored at -70°C until used. Quantitative gene expression was measured by real-time RT-PCR by applying a Rotorgene, RG3000 (Corbett Research) as described (Öhrvik et al., 2010) in the presence of 400 nM forward and reverse primers in a total volume of 12.5 μl (Table 1).

Western Blot

HC11 cells were seeded at a density of 50,000 cells/cm^2 in a total of 4 separate T75 tissue culture flasks and cultured and differentiated as described above prior to harvesting by

trypsination and homogenization of the obtained cell pellet in 5 volumes of RIPA-lysis buffer. HC11 cells in 2 T75 flasks were differentiated as described above while the cells in the other 2 flasks served as undifferentiated controls. The homogenization of the HC11 cells was carried out in 1.5 ml Eppendorf tubes by thorough pipetting. Homogenates were incubated on ice for 30 min and then centrifuged at 16,000Xg for 30 min at 4°C and supernatant protein concentrations determined as described (McKie et al., 2000). 20-50 μg of cellular protein was separated on a 10% Tris-Glycine polyacrylamide gel under reducing conditions andblotted to nitrocellulose as described (Öhrvik et al., 2007). Three nitrocellulose membranes with proteins from each T75 flasks were incubated in blocking buffer (5% nonfat dry milk powder in Tris buffered saline containing 0.05% Tween 20 (TBS-T) over night at 4°C. Membranes were then hybridized with a primary BCRP antibody (BXP-53, Abcam) diluted 1:100 or primary MDR1 antibody (JSB-1, Abcam) diluted 1:200 in TBS-T. The primary antibodies were detected by Horse Radish Peroxidase-conjugated secondary antibodies (ab6728, Abcam) diluted 1:7500 in TBS-T. In order to normalize the intensities of the bands, all the membranes were stripped at 60°C with buffer containing 62.5 mM Tris-HCl, 100 mM 2-mercaptoethanol, 2% SDS and then hybridized with anti-Tubulin antibody (YOL1/34) diluted 1:3000. Horse Radish Peroxidase-conjugated secondary antibodies to tubulin (ab6734, Abcam) diluted 1:5000 in TBS-T. HRP was detected by ECL Advance (GE Healthcare) and the intensities of the obtained bands quantitated as described (Öhrvik et al. 2010). As a quality control of the antibodies used in the Western with cellular protein, they were tested on protein isolated from mammary gland of pregnant and lactating mice.

Bcrp RNAi

Prior to the *Bcrp* RNAi of the HC11 cells optimization of transfection efficiency was performed by incubating various densities of HC11 cells with either siTOX Transfection

Control (Dharmacon) or a non-targeting negative control (On-Target plus siControl Non-Targeting Pool, Dharmacon) in the presence of various dilutions of DharmaFECT siRNA Transfection Reagents 1-4, as recommended by the manufacturer (Dharmacon). ATP levels were measured to check viability of the HC11 cells following each set of transfection condition by applying the ATP Determination Kit (Invitrogen), with formaldehyde-treated cells as positive controls. Firefly luciferase induced chemiluminescence of cellular ATP was measured on a Victor^2 1420 multilabel counter (PerkinElmer). Following the various transfection conditions the one resulting in the lowest viability following treatment with siTOX Transfection Control reagent and the highest viability following treatment with the On-Target plus siControl Non-Targeting Pool was selected. Following this optimization of transfection efficiency cyclophilin B siRNA transfection was performed under the optimized condition by the use of siControl cyclophilin B siRNA (targeting mouse cyclophilin, accession number NM_011149) according to the instructions of the manufacturer. The silencing of the cyclophilin B gene was measured by quantitative real-time RT-PCR with specific primers (Table 1) as described above. After successful silencing of cyclophilin B the optimized siRNA transfection protocol was adopted to silence *Bcrp* expression. HC11 cells were harvested in antibiotic-free RPMI 1640 medium and seeded into 6-well tissue culture plates at a density of 8,300 cells/cm^2. After incubation for 24 h at 37°C the medium was replaced with 2 ml of transfection medium (Opti-MEM) containing 100 nM On-Target plus SMART pool (targeting mouse BCRP, accession number NM_011920) and DharmaFECT 1 Transfection Reagent diluted 1:4. Mock transfected cells were incubated as described above with 2 ml Opti-MEM containing 100 nM On-Target plus siControl Non-Targeting RNA duplexes. Following transfection for 48 h at 37°C the HC11 cells were either subjected to RNA isolation for quantitative gene expression of *Bcrp* by real-time RT-PCR or transport experiments with MX (as described below).

Transport experiments

Prior to the experiments HC11 cells were seeded in 12 well plates at a density of 50,000 cells/cm^2 and cultured and treated as described above. MX (Sigma) was used to examine the function of BCRP in the HC11 cells using ^{3}H-MX, with a specific radioactivity of 4 Ci/mmol, as tracer (Moravek Biochemicals). It has previously been demonstrated that BCRP mediated transport of MX is reduced by GF120918 (de Bruin et al., 1999). Accumulation studies with MX were performed as follows. HC11 cells were rinsed with 2 x 1.5 ml of 37ºC Hank's Balanced Salt Solution with $CaCl_2$ and $MgCl_2$ (Invitrogen), pH 7.4 containing 25 mM N-(2-hydroxyehtyl) piperazine-N'-(2-ethanesulfonic) acid (HEPES, Sigma-Aldrich) (HBSS) and then pre-incubated for 30 min at 37º in 1.5 ml HBSS. After pre-incubation the HC11 cells were incubated at 37ºC for 60 min with 37ºC HBSS containing 1 μM MX supplemented 3000 Bq ^{3}H-MX/ml. Experiments including inhibitor were performed in HBSS, containing 1 μM of GF120918 during the 60 min incubation period. At the end of theexperiment the HC11-cells were rinsed with 3 x 1.5 ml ice-cold HBSS and thereafter lysed by adding 1 ml 0.5 M NaOH to each well. The MX concentration in the HC11 cells was then calculated from the radioactivity measurement by β-spectrometry using a 1900 CA Tri-Carb®Liquid Scintillation Analyzer (Packard Instruments). An aliquot of the cell lysate was used for protein determination by applying the BCA-method (as described above).

To study secretion of MX HC11 cells were preincubated as described above and then incubated with 1 μM MX supplemented 3000 Bq ^{3}H-MX/ml at 37ºC for 60 min. After this loading period the incubation medium was discarded and the HC11 cells were rinsed with 2 x 1.5 ml 37ºC HBSS. After the washes 1.5 ml of 37ºC HBSS with or without 1 μM GF120918 was added to the wells followed by incubation at 37ºC for 45 min. At the end of the secretion

experiment the HBSS was sampled and MX concentrations measured by β-spectrometry as described above. Both accumulation and secretion of MX was normalized to total cellular protein of the HC11 cells. Prior to the transport experiments potential cellular toxicity of the MX and GF120918 was assessed by measuring cellular ATP levels as described above or leakage of lactate dehydrogenase (LDH) as described previously (Aspenström-Fagerlund et al., 2007). No effects in ATP- or LDH-levels were detected in the experimental conditions and MX and GF120918 concentrations used in the present study indicating that the observed effects in accumulation and secretion were not a result of cytotoxicity induced by of these chemical compounds.

Statistics

Statistical analysis was performed using Statview 4.1 software for PC. Kruskal-Wallis was used to detect any significant differences in the data. Mann-Whitney was applied to examine statistically significant differences between two groups. The level of significance was set at $p \leq 0.05$.

Results

Cellular organization

The morphology of the HC11 cells was affected by the differentiation procedure. The differentiated cells showed a characteristic pattern with alveolar resembling structures including lumina, whereas the undifferentiated cells were organized as a monolayer (Figure 1).

mRNA expressions

Gene expression of β-casein (*Csn2*) and *Bcrp* was detected in both undifferentiated and differentiated HC11 cells. A statistically significant induction of β-casein gene expression was observed in the differentiated HC11 cells as compared to the undifferentiated controls (Figure 1a). Thus, the relative β-casein gene expression was increased 2-fold in the differentiated as compared to the undifferentiated HC11 cells (Figure 1a). The relative *Bcrp* gene expression was at a similar level in undifferentiated and differentiated HC11 cells (Figure 2a). A statistically significant 2-fold reduction in *Mdr1* gene expression was observed in the differentiated HC11 as compared to the undifferentiated ones (Figure 2b).

Protein expressions

Bcrp and Mdr1-protein was detected in both undifferentiated and differentiated HC11 cells (Figure 3). Tubulin normalized Bcrp protein expression was up-regulated and Mdr1 protein expression down-regulated in the differentiated as compared to the undifferentiated HC11 cells (Figure 3). Quantification of Bcrp and Mdr1 protein levels in differentiated and undifferentiated HC11 cells as performed in two separate experiments with triplicate analyses did not show any overlaps in protein levels.

Bcrp silencing

To test the function of our optimized transfection efficiency conditions RNAi was initially performed with the house-keeping gene cyclophilin B. Our results showed that almost a 90% down-regulation of the cyclophilin B gene expression was obtained (Figure 4). Using the same protocol with *Bcrp* siRNA-duplexes *Bcrp*-transcript levels were reduced to the same extent as cyclophilin (Figure 4).

Transport experiments

The transport experiments with ^{3}H-MX showed that the accumulation was increased in both undifferentiated and differentiated HC11 cells simultaneously incubated with GF120918 (Figure 5a). Thus, in the presence of the BCRP inhibitor the accumulation of MX increased with about 21% in the undifferentiated HC11 cells and with about 36% in the differentiated ones. Our results also showed that the accumulation of MX was higher in the differentiated HC11 cells as compared to the undifferentiated controls (Figure 5a). The presence of the inhibitor GF120918 also resulted in a decreased secretion of MX from the loaded HC11 cells (Figure 5b). The secretion of MX was reduced to about 65% in the presence of GF120918 in undifferentiated HC11 cells and to about 70% in the differentiated ones. The results obtained in the transport experiments with ^{3}H-MX showed that the accumulation of MX in HC11 cells increased 2-fold by *Bcrp* RNAi as compared to the MX accumulation in the mock transfected HC11 cells (Figure 5a).

Discussion

The results obtained in the present investigation demonstrate for the first time that both Bcrp and Mdr1 are expressed in the HC11 cells and are up- and downregulated, respectively by differentiation as in mammary epithelial cells *in vivo* during lactation. Thus, whereas Bcrp protein levels were increased in HC11 cells differentiated into a secreting phenotype, Mdr1 protein levels were decreased. These results are in line with the *in vivo* findings that BCRP is up-regulated and MDR1 down-regulated in lactating mammary glands in various species (Alcorn et al., 2002; Jonker et al., 2005; Gilchrist and Alcorn, 2009) and suggest that the HC11 model can be used to both assess function of endogenous Bcrp and to detect new substrates as well as inducers and inhibitors of this transporter.

HC11 cells have been used as a model for studies on hormonal regulation of mammary epithelial cell differentiation as well as milk protein gene expression and secretion (Wartmann et al., 1996; Desrivières et al., 2003; Kabotyanski et al., 2006). Furthermore, HC11 cells have been applied to examine the impact of toxic compounds on lactating mammary cells (Öhrvik et al., 2010) and also to characterize some transporters belonging to the Solute Carrier (SLC) family and ion channels, which are implicated in the flux of magnesium, zinc, copper and calcium across the membranes of the lactating mammary epithelium (Kelleher and Lönnerdal, 2005, 2006; Boyd and Náray-Fejes-Tóth, 2007; Öhrvik et al, 2010; Wolf et al., 2010; McCormick and Kelleher, 2012; Ross et al., 2013). However, the HC11 cell model has not yet been used to characterize expression and/or function of ATP Binding Cassette (ABC) transporters. The localization of both BCRP and MDR1 in the apical membranes of MECs in combination with the promiscuity of these active transporters has risen considerable concern from public health, food safety, and regulatory perspectives about the presence of drugs and

toxic compounds in milk both for breast fed infants as well as for consumers of dairy products (Wassermann et al., 2013).

Herein, we showed that *Bcrp* gene expression was at a similar level in undifferentiated and differentiated HC11 cells whereas *Mdr1* transcript levels were reduced in the differentiated ones. The reason for the lack of induction of *Bcrp* mRNA in the HC11 cells by lactogenic stimulation in the present investigation is unknown. However, one possible explanation may be that the HC11 cells originally derive from mammary tissue of BALB/c mice during gestation when *Bcrp* transcripts levels are induced to a stage where no further increases in gene expression occurs (Danielsson et al., 1984; Jonker et al., 2005). In support of this, silencing of *Bcrp* gene expression in undifferentiated HC11 cells resulted in a marked decrease in Bcrp function as assessed by increased MX accumulation, which probably would not have been possible to demonstrate if the endogenous Bcrp expression in the control cells was at a baseline level.

In the present investigation the HC11 cells differentiated by treatment with prolactin and cortisol were organized into alveolar-like structures with lumina resembling the organization of the mammary epithelial cells observed in the mammary glands *in vivo* during lactation (Burgoyne and Duncan, 1998; Richert et al., 2000).. Beside the changes in cellular organization by lactogenic stimulation β-casein gene expression was also induced in the HC11 cells. It has previously been demonstrated that β-casein responds to prolactin in HC11 cells (Ball et al., 1988) and that synthesis occurs in rodent MEC *in vivo* and that the abundance of this milk protein increases during gestation and peaks at mid-lactation (Robinson et al., 1995; Burgoyne and Duncan, 1998; Richert et al., 2000; McManaman and Neville, 2003). Thus, reorganization of HC11 cells into alveolar-resembling formations in

combination with an increased β-casein gene expression by lactogenic stimulation appears to be reliable markers of a secreting phenotype. Based on the results obtained in the present study reduced gene and protein expression of Mdr1 as well as increased expression of Bcrp protein may also be used as secretory markers of the HC11 cells.

Our results showed that the net accumulation of MX was higher in the differentiated HC11 cells as compared to the undifferentiated controls despite an apparent higher expression of Bcrp. This may, at least in part, be explained by a reduced expression of Mdr1. Although MX extrusion is predominantly mediated by Bcrp it has been demonstrated that MX can be transported to the extracellular compartment by Mdr1 although this transporter harbors less substrate specificity for MX than Bcrp and thus requires higher substrate concentrations (Ahmed-Belkacem, 2005; Rautio et al., 2006; Kodaira et al., 2010). Numerous SLC transporters, comprising both organic cation transporters (OCTs) and organic anion polypeptide transporters (OATPs), in MECs are affected at the transcriptional level by lactogenic stimulation (Alcorn et al., 2002; Gilchrist and Alcorn, 2010) including some with potential MX affinity. It may, hence, be possible that the increased accumulation of MX in the HC11 cells featuring a secretory phenotype as opposed to the undifferentiated ones is not only connected to the reduced expression of Mdr1 observed herein, but also to a concerted action of a number of other transporters including Oct or/and Oatp transporters.

Madine-Darby Canine Kidney II (MDCK II), human intestinal epithelial cells (Caco-2), human breast adenocarcinoma cells (MCF7), human choriocarcinoma cells (BeWo) and human bone osteosarcoma cells (SAOS-2) constitute examples of models used to characterize the function and substrate specificities of efflux proteins such as BCRP (Doyle et al., 1998; Jonker et al., 2000; Ceckova et al., 2006; Matsson et al., 2007; Li et al., 2011; Wasserman et

al., 2013). However, cancer cell lines and non-tumorigenic cell lines deriving from tissues other than the alveolar lining of the mammary glands harbor different sets of transporter protein panels at both the apical and basolateral membranes which may affect secretion and net accumulation of substrates may therefore differ (Krajci 2013). As expected by a Bcrp inhibitor, GF120918 treatment of HC11 cells decreased both net accumulation and secretion of MX. This corroborates our suggestion that the HC11 cells can be used for assessing transport of potential Bcrp substrates across the mammary epithelium.

The results obtained in the present investigation demonstrate for the first time that the murine mammary epithelial HC11 cells feature endogenous Bcrp and Mdr1 expression and that the expressions of these transporter proteins appear to respond to treatment with lactogenic hormones in a corresponding manner as mammary epithelial cells *in vivo* during lactation. Based on these findings it can be concluded that the HC11cells comprise a promising tool to assess the impact of drugs and other chemicals on endogenous BCRP function.

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Conflict of Interest

There are no conflicts of interest regarding this manuscript and the authors have nothing to disclose.

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4
5

Table 1. Exon-spanning oligonucleotide primers used for quantitative gene expression analyses by real-time RT-PCR.

Gene	Primer Sequences
β-casein	5'-CTTAACCCCACCGTCCAAT-3' (forward) 5'-AGCATGATCCAAAGGTGAAAA-3' (reverse)
Bcrp/Abcg2	5'-CGCAGAAGGAGATGTGTT-3' (forward) 5'-TTGGATCTTTCCTTGCTGCT-3' (reverse)
Mdr1/Abcb1	5'-GCTGTTAAGGAAGCCAATGC-3' (forward) 5'-AGCAATGGCGATTCTCTGGTT-3' (reverse)
Cyclophilin B	5'-GCGCAATATGAAGGTGCTCT-3' (forward) 5'-GAAGTCTCCACCCTGGATCA-3' (reverse)

Legends to the figures

Figure 1. a. Cellular organization of undifferentiated and differentiated HC11 cells as described in Materials and Methods. Undifferentiated HC11 cells not stimulated with lactogenic hormones are organized as monolayers (left). Differentiated cells treated with prolactin and hydrocortisone are formatted in alveolar-resembling structures with alveolar lumina (right). b. Relative β-casein gene expression in undifferentiated and differentiated HC11 cells, as described in Materials and Methods. The data are presented as means ± SD of 6 samples from 2 separate experiments. *Statistically significant different as compared to undifferentiated controls, $p \leq 0.05$.

Figure 2. Relative gene expression of *Bcrp* (a) and *Mdr1* (b) in undifferentiated and differentiated HC11 cells, as described in Materials and Methods. The data are presented as means ± SD of 6 samples from 2 separate experiments. *Statistically significant different as compared to undifferentiated controls $p \leq 0.05$.

Figure 3. Protein expression of BCRP (a) and MDR1 (b) in undifferentiated and differentiated HC11 cells, normalized to tubulin expression as described in Materials and Methods. The data represent BCRP and MDR1 protein expression in HC11 cell lysates obtained in two separate experiments, each comprising three membranes per protein expressed as relative means and ranges.

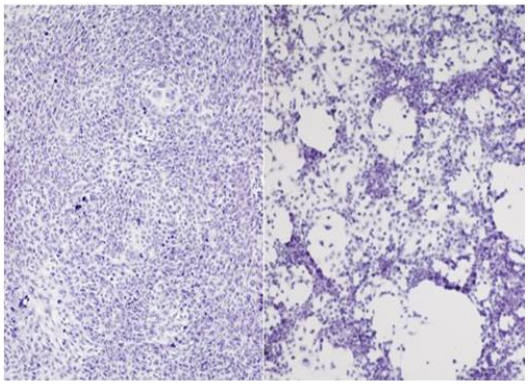
Figure 4. Relative gene expressions of cyclophilin B and *Bcrp* in HC11 cells subjected to RNA interference (RNAi). The data are presented as means ± SD of 3-5 samples obtained from 2 separate experiments. *Statistically significant as compared to untransfected controls

(Opti-MEM) and mock transfected cells (Opti-MEM containing Lipid 1 diluted 1:4 and non-targeting RNA duplexes) as described in Materials and Methods, $p \leq 0.05$.

Figure 5. Accumulation (a) and secretion (b) of ^{3}H-mitoxantrone in undifferentiated (No) or , differentiated (Yes) HC11 cells, treated with the BCRP inhibitor GF120918, and in mock transfected (mock) and *Bcrp* RNAi treated (Bcrp RNAi) HC11 cells, as described in Material and Methods. The data are presented as means ± SD of 6-12 samples and expressed as pmol mitoxantrone/mg cellular protein as described in Materials and Methods. Statistically significant differences between groups are indicated by links between the columns (*$p \leq 0.05$; **$p \leq 0.01$).

Figure 1.

a



b

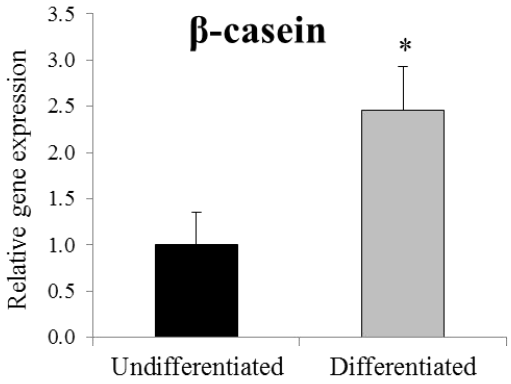

β-casein
Relative gene expression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Undifferentiated
Differentiated

Figure 2.

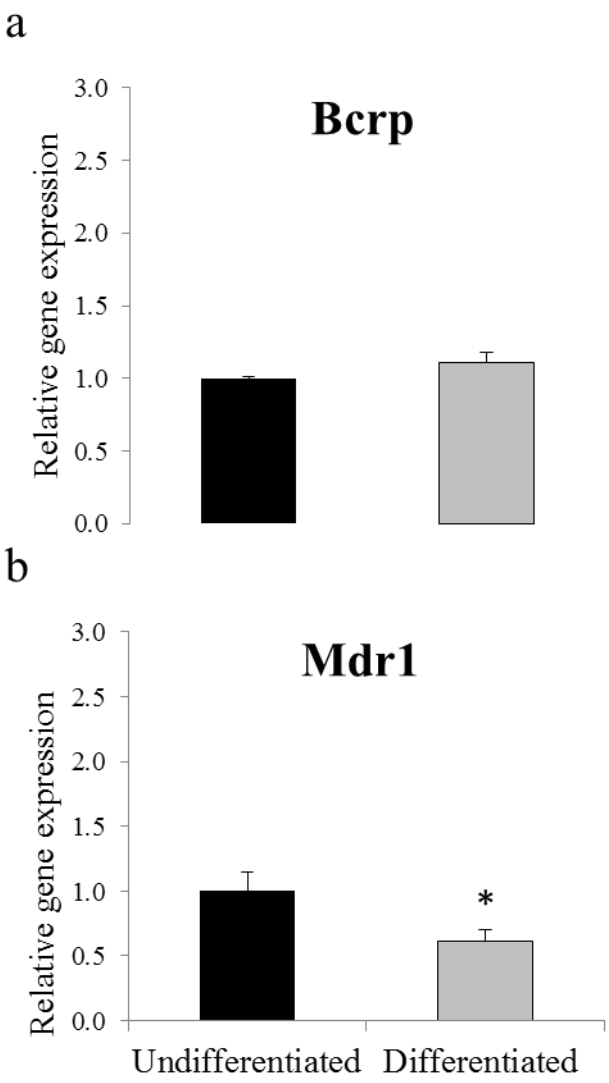

a
Bcrp
Relative gene expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
b
Mdr1
Relative gene expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Undifferentiated
Differentiated

Figure 3.

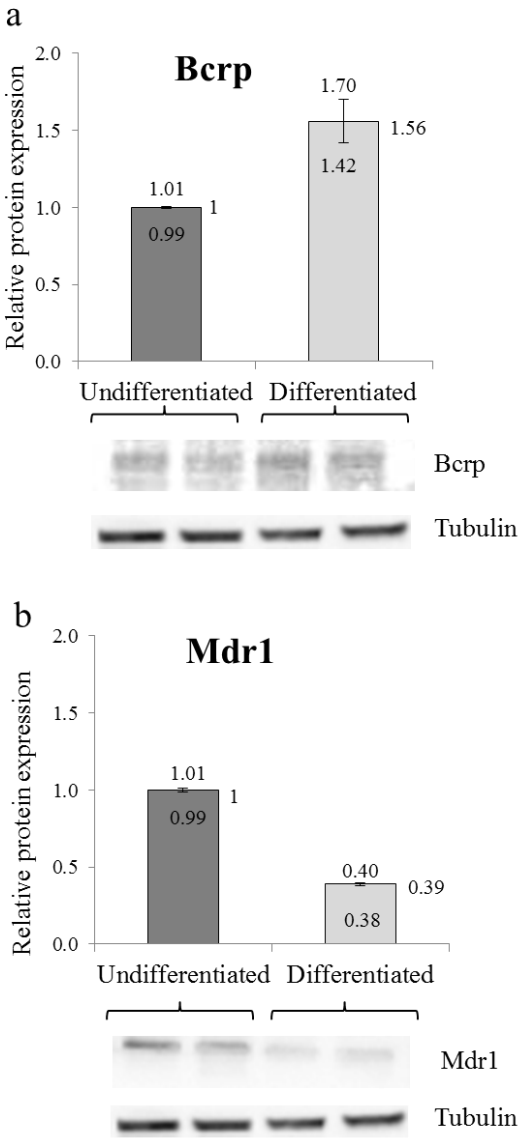

a
Bcrp
Relative protein expression
2.0
1.5
1.0
0.5
0.0
1.01
1
0.99
1.70
1.56
1.42
Undifferentiated
Differentiated
Bcrp
Tubulin
b
Mdr1
Relative protein expression
2.0
1.5
1.0
0.5
0.0
1.01
1
0.99
0.40
0.39
0.38
Undifferentiated
Differentiated
Mdr1
Tubulin

Figure 4.

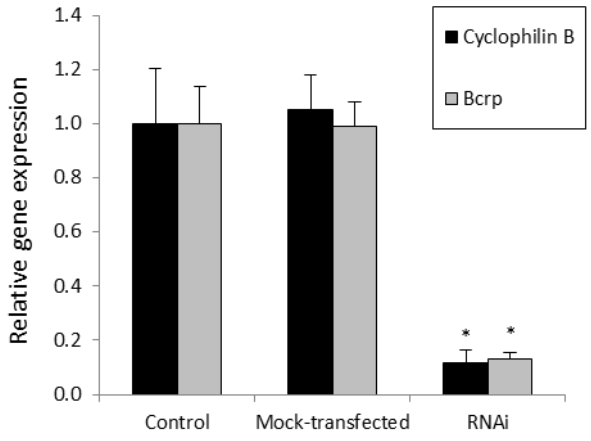
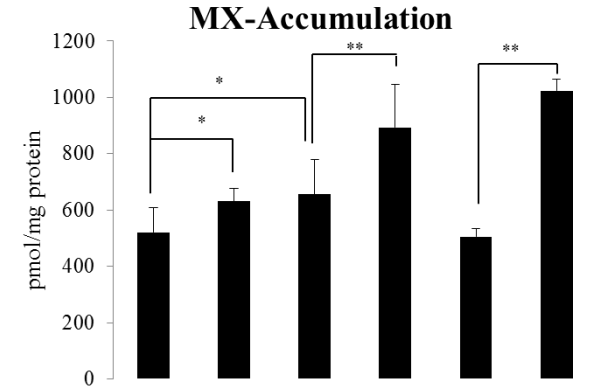

Relative gene expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Cyclophilin B
Bcrp
*
*
Control
Mock-transfected
RNAi

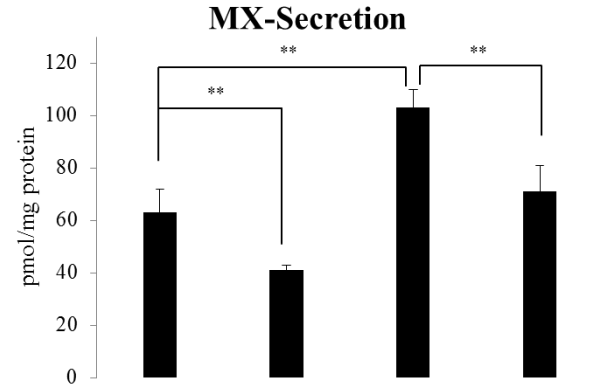
Figure 5.

a



Differentiated	No	No	Yes	Yes	No	No
GF120918	-	+	-	+	-	-
Mock	-	-	-	-	+	-
Bcrp RNAi	-	-	-	-	-	+

b



Differentiated	No	No	Yes	Yes
GF120918	-	+	-	+

1