Transporter protein expression and localization in murine mammary epithelial HC11 cells

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ABSTRACT

Milk is the sole food for breast-fed infants and a significant source of nutrition for consumers of dairy products. It has a very complex composition containing both macronutrients and micronutrients needed for growth and development. Drugs, carcinogens and environmental toxins can be secreted in milk posing a potential health risk for breast-fed infants and milk consumers. Active transport by membrane proteins in secretory mammary epithelial cells can explain this process. Several membrane protein transporters from different protein families (Solute carrier (SLC) and ATP-binding cassette (ABC) super families) mediate transport of a wide range of different compounds.

The major purpose of this project was to investigate transporter protein expression of mouse Bcrp, Mdr1, Octn1 and Oatp3 (Oatp1a5) by using a murine mammary epithelial HC11 cell line. Differentiated HC11 cells featuring a milk protein synthesizing and secreting phenotype were used in western blot experiments to detect the proteins of interest. In this cell model it was possible to detect upregulation of membrane transporters mouse Bcrp and Octn1 as a result of the differentiation. In addition, immunohistochemistry on lactating mouse mammary gland tissues was performed to detect the localization of mouse Octn1. Octn1 was localized at the apical membrane of mammary epithelial cells supporting the theory that it plays a role in the transport of carnitine into milk. The impact of lipopolysaccharide (LPS) treatment, mimicking mammary inflammation, on protein expression of transporters in secreting HC11 cells were examined by western blotting. However, at the LPS concentration used no effects were detected.

This study provides basic tools for understanding the nature of secretion of nutrients and nonnutrients to milk by membrane transporters. Further investigations are required to understand the correlation between protein expression and function of membrane transporters in secreting HC11 cells.

CONTENTS

1. INTRODUCTION

1.1 Milk is a nutrient source and a source of contaminants for the newborn

Milk is the sole food for breast-fed infants and a significant source of nutrition in consumers of dairy products. It has very complex composition containing both macronutrients and micronutrients needed for growth and development. It is a nutrient source of proteins, lipids, vitamins, amino acids, minerals as well as hormones, growth factors and enzymes (Haug et al., 2007). In addition to this unique advantage of breast milk; drugs, carcinogens and environmental toxins also can be secreted in milk (Jonker et al., 2005). When at high milk-to-serum (M/S) ratio is observed in animal experiments studies especially for drugs, their accumulation into milk may involve active transport by transporter proteins (Alcorn et al., 2002). Thus, contamination of milk with such xenobiotics and drugs poses a potential health risk for breast-fed infants and dairy consumers.

1.2 Mammary gland development in mouse during puberty, pregnancy, lactation and involution

Embryonic development of mammary gland starts to appear by day 12. Because of the fast proliferation of epithelial cells mammary epithelium gains a new shape by day 14 and continues by day 16 forming mammary sprout. This sprout grows into ductal tree which consists of several branching ducts until birth (Richert et al., 2000). Hormones are continuously involved in all stages of mammary gland development (Meyer et al., 2004). Estrogen and growth hormones are responsible for the ductal morphogenesis (Neville et al., 2002). Postnatal development occurs after embryonic development has ended and consists of two parts. In puberty ductal growth has roles in lengthening and branching of the epithelium. Alveolar differentiation appears during pregnancy and alveolar buds become responsible units for the milk secretion (Richert et al., 2000). The proliferative phase of alveolar morphogenesis needs prolactin and progesterone hormones to be able to start differentiation (Neville et al., 2002).

Lobular alveolar structures grow during pregnancy and the mammary gland is converted to exocrine organ from a simple ductal tree. This process creates a lot of changes including hormonal changes in the cellular composition of the mammary gland. To be able to achieve successful transport and secretory functions, some biochemical properties also change during pregnancy (Meyer et al., 2004). Lobuloalveolar phase of the mammary growth takes place at

the beginning of the pregnancy when the alveolar buds start to divide and differentiate into individual alveoli which are responsible for milk secretion during lactation. To produce milk, the alveoli expand through the fat pad and the lumen is filled by milk containing milk proteins and lipids. When lactation starts, the mammary gland is totally filled by alveoli. Lactation continues for about 3 weeks in mice when the pups are weaned (Richert et al., 2000).

Following weaning milk synthesis in the mammary epithelial cells stops and involution and remodeling of mammary tissue takes place. At the end of the involution the mammary gland is observed as a prepregnant resting gland (Meyer et al., 2004).

1.3 Hormone dependent proliferation and differentiation stages of HC11 cells

HC11 mammary epithelial cells can be used as a model for investigating cellular differentiation which is regulated by hormones (Desrivières et al., 2007). Since this epithelial cell line preserves the characteristics of normal mammary epithelial stem cells, it is still being used as a well-established in vitro system in studies related with gene and protein expression (Desrivières et al., 2007). Under the control of lactogenic hormones this cell line is able to synthesize the major mouse milk protein beta casein (Doppler et al., 1989). The origin of the cell line was emerged from mid-pregnant BALB/c mouse mammary gland tissue (Danielson et al., 1984). This regulatory function of the hormones shows synergistic effect to induce differentiation from a proliferative to differentiated stage (Desrivières et al., 2007). Janus kinase (Jak) - signal transducer and activator of transcription (Stat) pathway (Jak/Stat pathway) is activated by prolactin thereby inducing differentiation of the cells (Pauloin et al., 2012). Stat 5 is a signaling factor for transcription which takes place in protein- protein interactions with glucocorticoids and mediates the prolactin response (Reichardt et al., 2001). Transcription of the beta-casein which is the marker of mammary gland differentiation is activated by the glucocorticoids action by the physical interaction between its receptor and Stat5 (Desrivières et al., 2007). The growth of the epithelial cells until they become confluent occurs in the presence of insulin and epidermal growth factor (EGF) but glucocorticoids start the differentiation working together with insulin and prolactin (Shan et al., 2008). The synthesis of milk proteins including caseins, whey acidic protein and α-lactalbumin is induced by prolactin in synergy with glucocorticoids (Pauloin et al., 2012). It has been shown that estrogens are needed for the development of ductal system after birth while progesterone is needed during pregnancy for the lengthening and branching of ducts and for the growth of lobuloalveolar system (Reichardt et al., 2001).

1.4 Transport mechanisms in mammary gland

There are a lot of activities taking place in mammary gland including passive diffusion and active transports of various substances. There are five major pathways for both transport of substances and secretion of milk protein, lipids, nutrients and ions into milk. *The exocytotic pathway* stands for the milk protein secretion and also for oligosaccharides and various nutrients that are packaged into secretory vesicles and transported to the apical region of the cells. When these vesicles leave the Golgi and reach to the apical plasma membrane, they complete their function by merging the content into the extracellular space. *Lipid secretion pathway* is unique to mammary epithelial cells and basically lipids and lipid- associated proteins are transported to the apical plasma membrane as membrane enveloped structures called milk fat globules. *Transcytotic pathways* work on transport of the macromolecules starting from the basal membrane to the apical membrane. Immunoglobulin A and prolactin can be given as example substances for this type of transport. *Membrane transport pathway* contains lots of different mechanisms for ions, glucose and amino acids. The movement of these substances from blood to the milk is a kind of transcellular transfer and to be able to complete the transport; specific transporters are needed at the basal and apical plasma membranes which have roles in active transport in alveolar cells. Unlike passive diffusion active transport is not dependent on concentration gradient. It is believed that active transport mechanisms took place when certain drugs' concentrations are present in milk higher than expected. Passive diffusion can be predicted by physical characteristics of the compounds while active transport cannot. *The paracellular transport* is responsible for the transport of substances that have low molecular weight and also for the macromolecules. But mammary epithelial cells have tight-junctions between each other except during pregnancy, involution and in inflammatory states as mastitis. These tight junctions are responsible to control paracellular exchange of any kind of substances (McManaman et al., 2003).

1.5 ATP- binding cassette transporters

The ABC transporter superfamily members are known as responsible transporters by an energy dependent manner and they are believed to efflux molecules in the presence of ATP binding or hydrolysis (Robey et al., 2011). Some recent and interesting studies have been done with these transporters on contamination of milk with drugs, pesticides and other xenotoxins (Jonker et al., 2005). Two important efflux membrane proteins are heavily involved in transport of these exogenous compounds: ABCB1/P-glycoprotein (P-gp)/MDR1 and ABCG2/breast cancer resistance protein (BCRP) (Krishnamurthy et al., 2006). The breast

cancer resistant protein BCRP is encoded by the *ABCG2* gene and the multidrug resistance protein (P-gp) is encoded by the *ABCB1 (or MDR-1)* (van Herwaarden et al., 2006b)

1.5.1 Breast cancer resistance protein (Bcrp/Abcg2)

BCRP was first cloned from a MCF7 multidrug-resistant breast cancer cell line and its name comes from there (Staud et al., 2005). By radiation hybrid analysis, Allikmets et al. (1998) mapped the ABCG2 gene to human chromosome 4q22, between markers D4S2462 and D4S1557. The mouse Bcrp1 is very similar to human BCRP. Because of that reason numerous functional BCRP experiments have been performed in mice (van Herwaarden et al., 2006a). BCRP has a 72-kDa molecular weight with 665 amino acids and it has ATP- binding domain at N-terminal as well as six transmembrane domains (TMD) at the C-terminal which are responsible for drug binding and efflux of the substrates (Staud et al., 2005). Substrates cross membranes by using ATP and this is provided by nucleotid binding domain (NBD) and it is believed that these domains are highly conserved (Krishnamurthy et al., 2006). Localization of the BCRP has been shown several times in different studies by immunohistochemical detection and concluded to be at the apical side in most of the mammalian tissues such as epithelium of the intestine, kidney, placenta, and the bile canalicular membrane and also in mammary gland (Staud et al., 2005 and Jonker et al., 2005). While BCRP has a protective function in most of tissues in the body by controlling disposition of the substrates and protecting body by pumping xenobiotics out, in the mammary gland this protective function turns out a harmful function. Since it is upregulated in the lactating mammary gland of mice and humans, it can transport high amounts of BCRP substrates like PhIP (2-amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine) from mother into the milk (Robey et al., 2011). The upregulation of Bcrp at the protein level has also been shown in mouse mammary gland epithelium during late pregnancy by western blot analysis (Jonker et al., 2005).

1.5.2 Multidrug resistance protein 1 (Mdr1/P-gp)

Another important member of the ATP-binding cassette (ABC) superfamily that has been studied extensively is P-glycoproteins (Schinkel et al., 1997). The gene coding for P-gp in humans was first isolated and cloned in the 1980s. The protein has 170-kDa molecular weight and the protein structure is like many other ABC proteins (Leonessa et al., 2003). It has two membrane binding domains that contain six transmembrane (TM) helices for each and two

cytoplasmic nucleotide-binding (NB) domains which have roles in binding and hydrolyzing ATP (Jones et al., 2004). Some studies have showed that Pgp protein is present and expressed at low levels in most tissues but it is mostly localized at the apical surface of the epithelial cells in liver, colon, kidney and pancreatic ductules (Thiebaut et al., 1987). Because of its localization in many tissues it is believed that Pgp plays a role in the protection of the organs by secreting of metabolites and xenobiotics out of the epithelial cells (Schinkel et al., 1997). Beside these tissues, some studies at RNA level of transporter genes in the lactating mammary epithelial cells has showed that *MDR1* is expressed in mammary epithelial cells and unlike *BCRP*, it is down regulated in lactating mammary epithelial cells during lactation (Alcorn et al., 2002). The down regulation of Mdr1 at the protein level has also been shown in mouse mammary gland epithelium during late pregnancy by western blot analysis (Jonker et al., 2005).

1.6 Organic cation transporters (Solute carrier transporters)

The mammary gland is known as milk-producing organ but it is not the only useful characteristic of it. It mediates a stabilized transfer of compounds by increasing nutrients and endogenous bioactive factor accumulation in milk and by restricting transfer of toxic substances and nonnutritional compounds. It was believed that this transfer was caused by ion trapping or passive diffusion. Although the molecular mechanisms are not fully understood, some studies have shown accumulation or excretion of these organic cations in milk are higher than expected from passive diffusion and ion trapping (Kwok et al., 2006)

There are a lot of studies of membrane transporters belonging to the organic cation transporters (OCT) family in various tissues and also in different species which have been shown that this transporter family has important roles in the absorption, distribution and elimination of various cationic drugs, xenobiotics and endogenous metabolites or zwitterions (Lahjouji et al., 2001) These proteins are classified as the solute linked carrier family (SLC).

SLC are responsible for the transfer of specific compounds into epithelial cells across the apical membrane. These transporters have wide substrate specificity (Sugiura et al., 2010). L-carnitine is the most important and essential substrate for solute carrier transporters that have been investigated in many studies and the relationship between them is still being investigated. L-carnitine is a small water-soluble zwitterion, have critical physiological roles as a cofactor that involves in the β-oxidation of long chain fatty acids and glucose oxidation in the muscle and heart for ATP generation. Carnitine generates energy for brain in the

development of the nervous system. Because of its function in many different tissues but especially in early brain growth and development, carnitine delivery via breast milk to the newborn can be considered a major issue during lactation. (Lamhonwah et al., 2011)

It has been shown that carnitine is transported by a family of three carnitine/organic cation transporters; Organic Cation Transporter, Novel Protein, Type1 (OCTN1), Type 2 (OCTN2) and Type 3 (OCTN3) (Lamhonwah et al., 2011). It was first cloned from human fetal kidney (Tamai et al., 1997) and is expressed in different tissues in mouse such as intestine, liver (Sugiura et al., 2010) and mammary gland (Kwok et al., 2006). Mouse Octn1 has 553 amino acids and it works as a Na+ -dependent carnitine transporter. The comparison by Alcorn et al., (2002) of RNA expression levels of transporters in lactating and nonlactating mammary epithelial cells has shown that the gene expression of human *OCTN1* upregulated during lactation.

1.7 Organic anion transporters (Solute carrier transporters)

Membrane solute carriers are responsible for mediating the sodium independent transport. It occurs in bidirectional manner by receiving some organic compounds into cells and pumping bicarbonate and glutathion out of the cells. These transporters are expressed in different tissues such as choroid plexus, blood brain barrier (BBB), lung, heart, intestine, placenta, testis, kidney and testis. Organic Anion Transporting Polypeptides (Oatp/OATPs) are a group of this transporter family and have very extensive range of amphipathic organic substrates including bile salts, thyroid hormones, organic dyes, anionic oligopeptides, different drugs and xenobiotic compounds. There was already one old and traditional classification for this family but since this classification system doesn't give accurate and species independent identification, researchers have created a new species-independent classification and nomenclature system which was divided into families, subfamilies, individual genes and gene products based on the similar amino acid sequence identities (Hagenbuch et al., 2004)

Solute carrier organic anion transporter family member 1A2 (OATP1A2) [previously called anion transporting polypeptide A (OATP-A)/ (SLC21A3)] was the first human superfamily member and it was isolated by hybridization screening from human liver. OATP1A2 has four homologs within the subfamily OATP1A and the one that we are interested in is solute carrier organic anion transporter family member 1A5 (Oatp1a5) [previously called Organic Anion Transporting Polypeptide 3 (Oatp3)/ (Slc21a7) (Hagenbuchet al., 2003). Radiation hybrid analysis has demonstrated the human OATP-A gene has been localized on chromosome 12

and it was shown that OATP-A is the human ortholog of mouse Oatp3 (Oatp1a5) by fluorescent in situ hybridization studies (Walters et al., 2000). Generally the molecular weight for the Oatps/OATPs protein family can differ between 60 and 90 kDa. Basically they all have 12 transmembrane domains with C terminal and N terminal that are localized at the intracellular side (Hagenbuchet al., 2003). Although some Oatps/OATPs are selectively expressed (only in a single tissue), many of them show multiple tissue expression. Based on its function on the tissue Oatps can be localized at the basolateral side of the cell as well as at the apical side (Hagenbuchet al., 2004). Hagenbuchet al., (2004) has shown that some Oatps appears to be expressed in different sides of the epithelial cells in different organs in rat while Oatp3 (Oatp1a5) shows consistent apical expression in polar epithelial cells. The comparison of RNA expression levels of each transporter in lactating and nonlactating mammary epithelial cells have been shown by Alcorn et al., (2002) and according to this research the gene expression of OATPA which is the homologous gene of Oatp3 is upregulated during lactation (Hagenbuchet al., 2003).

1.8 Simulation of bovine mastitis using HC11 cells and lipopolysaccharides (LPS)

Mastitis stands for inflammatory reaction of the udder tissue in bovine mammary gland. When the pathogens invade teat canal of the mammary gland, the number of the white blood cells (leucocytes) increases to prevent damage caused by inflammation. 137 different organisms were identified as a cause of mastitis including bacteria, mycoplasma, yeast and algae. Since most of the mastitis pathogens are bacterial origin, *Staphylococcus aureus* and *Escherichia coli* seemed to be major bacterial pathogens which are responsible for bovine mastitis (Bradley et al., 2002). Little information is available about impacts of subclinical mastitis on active transport of chemicals into milk. Lipopolysaccharide (LPS) is an endotoxin found in the outer membrane of Gram-negative bacteria, such as S. aureus and E.coli. It would be interesting to examine the effect of LPS on protein expression of transporters in secreting HC11 cells. There are some studies related with Octn1 regarding the effects of inflammation on L-carnitine transporter expression in the mammary gland. It is believed that transporter expression and function might change during differentiation and it may depend on different lactation stages in the mammary gland. Ling et al., (2010) has showed the upregulated expression levels without statistical significance of L-carnitine transporter Octn1 in the mammary gland tissue when the lactating rats were treated with lipopolysaccharide (LPS) at different lactation stages.

2. AIMS OF THE STUDY

The major purpose of this project was to investigate transporter protein expression of mouse Bcrp, Mdr1, Octn1 and Oatp3 (Oatp1a5) by using a murine mammary epithelial HC11cell line. To be able to use this cell line, the differentiation of the HC11 cells into a milk protein synthesizing and secreting phenotype need to be achieved. Western blotting techniques were performed to detect the proteins of interest. In addition, immunohistochemistry on lactating mouse mammary gland tissues was performed to detect the localization of mouse Octn1. Second aim was to examine impact of lipopolysaccharide (LPS) inflammation on protein expression of transporters in secreting HC11 cells.

3. MATERIALS AND METHODS

3.1 HC11 cell culturing

Murine mammary epithelial cell line HC11 was used for the determination of transporter proteins' expression levels. The cells were grown in sterile filtered RPMI 1640 medium supplemented with 2mM L-Glutamine, 25 mM HEPES (Gibco, Invitrogen, Carlsbad USA), gentamicin 50 µl/ml (Gibco, Invitrogen, Carlsbad USA), 7.5 % sodium bicarbonate, bovine insulin 5 mg/l (Sigma-Aldrich, St Louis, USA), EGF (epidermal growth factor)-5µg/l and 10% heat-inactivated foetal bovine serum (FBS) (Gibco, Invitrogen, Carlsbad USA) at a pH between 7.3 and 7.4. The culture was grown at 37°C in 5% CO_2. Culture medium was changed every 2-3 days.

When the cells had reached 80-90% confluence, they were passaged:

The culture medium was aspirated from the flask and the cells were washed in sterile phosphate buffered saline (PBS) solution (15ml/75cm^2 or 5ml/25cm^2). PBS was removed and 3ml/75cm^2 or 1ml/25cm^2 Cell Dissociation Solution (Gibco, Invitrogen, and Carlsbad, CA, USA) was added and followed by incubation for 10-20 minutes at 37°C in 5% CO_2. When the cells were detached, they were suspended with 12ml/75cm^2 or 4ml/25cm^2 of culture medium, transferred to a falcon tube and spinned down at 450xG for 5 minutes at 4°C in a GS-GR centrifuge (Beckman Coulter, Fullerton, USA). The supernatant was removed carefully and the cell pellet was resuspended in a suitable amount of medium by pipetting up and down for several times. The cell suspension was transferred into new flasks prefilled with warmed growth medium.

Differentiating Cells (Protocol I)

Cells were cultured in growth medium for 3-4 days post confluency. After this, cells were stimulated to differentiate by exchanging growth medium with differentiating medium (growth medium – EGF, -FBS, + prolactin (Sigma-Aldrich, St Louis, USA) 1 µg/ml, +cortisol (Sigma-Aldrich, St Louis, USA) 1µM). The cells were grown in differentiating medium for 3-4 days in $25cm^2/75cm^2$ flasks. Two/three bottles of differentiated cells were grown in differentiated medium and two/three bottles of undifferentiated cells were grown in regular growth medium, as controls.

Differentiating Cells (Protocol II)

The HC11 cells were cultured in growth medium for 6 days post confluency. After this cells were washed with PBS and maintained in growth medium without EGF for 24 hours. To induce lactogenic differentiation the cells were incubated in differentiation medium for 3-4 days as described above.

3.2 Treatment of HC11 cells with Lipopolysaccharide (LPS)

The differentiation medium was aspirated from differentiated cells and the cells were washed with DMEM ($15ml/75cm^2$). To induce experimental inflammation cells were incubated at 37°C in 5% CO_2 in the presence of 10µg LPS/ml for 2 days. Control cells were incubated in LPS-free medium. LPS treatment was used to mimic mastitis in HC11 cells.

3.3 Lactate Dehydrogenase (LDH) cell toxicity test to LPS treated differentiated HC11 cells

1 ml of the medium was taken from both the lipopolysaccharide treated cell culture and the non-treated control cell culture just prior to protein isolation (as described below). 4.65 ml Tris HCl buffer (0.2mM pH 7.3) and 175µl of the NADH stock solution (6.6 mM) were mixed and vortexed shortly. 195µl of this diluted NADH solution was added into each well in a 96-well plate. 10 µl duplicate samples from the cell toxicity test per well were used. Two different control samples were used including low LDH and high LDH samples from previous experiments and the microplate was covered with parafilm, incubated at room temperature for 15-20 minutes. Then microplate was put in a microplate reader and incubated at 25°C for another 5 minutes. After incubation 15µl of the Na-pyruvate solution (30mM)/well was added by using a multichannel pipette without taking the microplate out. Absorbance values 340nm

were recorded (6 times for each well) and LDH concentrations calculated by applying the following formula:

LDH (U/ml) = ΔA_{340nm} per min/ (0.001 x 0.1 x lightpath (cm))

3.4 Isolation of proteins using RIPA-Buffer

The culture medium was aspirated and the cells were washed in sterile phosphate buffered saline (PBS) solution (15ml/75cm^2 or 5ml/25cm^2). PBS was removed. The cells were detached from the flasks by treatment with 1.5ml of trypsin and the flasks were inverted gently to assure that trypsin has reached to all cells. Trypsin was aspirated and the cells were incubated for 5 minutes at 37°C in 5% CO_2. Trypsin was neutralized by addition of 10 ml of basal medium containing 10% FBS. Cells were suspended; they were transferred to a falcon tube and spinned down at 450xG for 5 minutes at 4°C in a GS-GR centrifuge (Beckman Coulter, Fullerton, USA). The supernatant was removed carefully and the cell pellet was solved in 250/500µl RIPA buffer (Radio Immuno Precipitation Assay buffer- containing 47.5 ml 1X TE buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA), 0.4383 g NaCl, 0.5 g NP-40 (nonylfenoxy-polyetoxy-ethanol; Tergitol) 0.5 ml 10% SDS (sodium dodecyl sulphate), 0.5 tablet Complete protease inhibitor cocktail (Roche), 1X TE buffer up to 50 ml). The cell suspension was pipetted up and down repeatedly until a homogenate solution was obtained.

Mammary tissues were obtained from previous experiments with lactating mice approved by the Local Ethics Committee of Animal Research (permit no. C159/2). Tissues were homogenized with the suitable amount of RIPA buffer (0.1µg/µl) by using plastic homogenizer.

The homogenates were transferred into new eppendorf tubes and were then incubated on ice for 30 min followed by centrifugation for 30 min at 16000 x g. Supernatants were transferred carefully to new eppendorf tubes without touching the cell pellet. The isolated proteins were stored at -70 °C for further application

3.5 Determination of protein concentration

BCA assay was performed and bovine serum albumin (BSA) was used as protein standard. Dilutions for standard protein (concentration of BSA was 2mg/ml and the final volume was 250ul):

Table 1 Concentration of Standards for BSA assay

BSA (mg/ml)	BSA (µl)	RIPA Buffer (µl)
0	0	250
0,025	3,125	246,875
0,05	6,25	243,75
0,1	12,5	237,5
0,3	37,5	212,5
0,5	62,5	187,5
0,7	87,5	162,5
1	125	125

The first 3 columns in 96-well plate were used for the standard calibration curve. It was done in triplicates according to the above table. The next columns were used for unknown samples, triplicates for control samples and triplicates for differentiated samples. BCA Protein Assay Reagents were prepared by mixing 0.2 ml coppersulphate (reagent B) and 9.8 ml BCA (reagent A). A green solution formed. 190 µl of this mixture and 10 µl of each sample were added to each well. The well plate was covered with parafilm and incubated at 37°C for 30 minutes in the incubator oven. The absorbance was measured at 570nm by applying a microplate reader. Amount of protein in each well was calculated by plotting a standard curve on Excel. The protein concentrations were determined based on the equation for each of the unknown samples and their concentration units were the same as standard.

3.6 Gel electrophoresis

Sodium Dodecyl Sulfate PolyAcrylamide Gels (SDS-PAGE) were used to separate proteins according to their molecular weight. The protein expression of mouse Bcrp, Mdr1, Oatp3 (Oatp1a5) and Octn1 was examined in the control and differentiated cells of HC11 cell line

and also in mammary gland tissues from non-lactating virgin and lactating day 7 mouse as positive controls. In all experiments two control samples (control 9 and control 10) and two differentiated samples (differentiated 9 and differentiated 10) were used originating from different T75 flasks but grown at the same time. Western blotting with the samples from LPS treatment was done by using the same protein extraction. Protein samples were prepared boiling 5 minutes at 95°C in laemmli buffer with 1:1 raito with samples (Laemmli buffer was prepared with 950µl of laemmli and 50µl of betamercaptoethanol). BioRad Mini-PROTEAN Ready Gels (10%, 10-well comb, 50µl wells) was placed into the electrophoresis tank. The tank was filled with SDS-PAGE running buffer (10x Tris/Glycine/SDS buffer). Samples were loaded into each well using a long thin pipette tip. 7.5µl loading standard was added as molecular weight markers and 15-40 µl (20-50µg of protein) of each sample was loaded onto the gel. The tank was closed with the lid and electrodes were connected to the power supply. The gel was run at 30mA at room temperature until the loading dye had reached to the bottom of the gel. The gel was removed from the plastic covers and incubated in transfer buffer (Methanol and Tris glycine buffer) for 20 min. The gel was used for western blotting or Coomassie blue staining.

Coomassie blue staining

SDS-PAGE gel was removed from plastic plates and rinsed in milliQ water in a suitable container. Coomassie stain was added enough to cover the gel and it was incubated for 1 hour on the shaker. Then Coomassie Stain was poured off and the gel was rinsed twice with water to remove Coomassie Stain from the container. Destain solution was added enough to cover the gel and it was renewed every 30 minutes until the proteins bands became visible. An imaging system the Bio-Rad Molecular Imager ChemiDoc XRS was used for documentation of the gel. The Figure 1 shows the separation of the proteins.

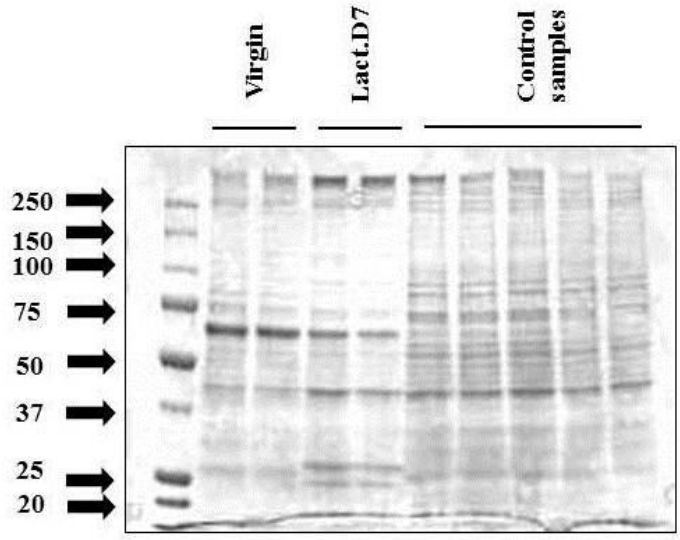

Figure 1 HC11 cell proteins from control samples and proteins from mouse virgin and lactating day 7 were separated on SDS-PAGE gel and visualized with Coomassie Staining.

3.7 Protein transfer to nitrocellulose membrane

The proteins were separated by SDS-electrophoresis and the SDS-PAGE gel was transferred to nitrocellulose membrane (0.45 um pore) by electrophoretic transfer by using the BioRad Mini Trans-Blot Electrophoresis Transfer Cell. All the materials in the blocking sandwich were incubated in transfer buffer which consisting of sponge, filter paper, SDS-PAGE gel, nitrocellulose membrane, filter paper and sponge. They were placed in the transfer cassette with the gel on the side of negative electrode (black) and the membrane on the side of the positive electrode. Protein transfer was performed at 350 mA for 1.25 hour. During the blotting procedure an ice bar was out in the buffer tank and the whole tank was placed in a box full of ice to keep the temperature of the transfer buffer below 40°C

3.8 Detection of proteins on a Western Blot by Chemiluminescent Detection Kit

After the transfer of proteins from gel to a nitrocellulose membrane, the membrane was incubated in 50 ml of blocking buffer (5% nonfat dry milk powder in Tris based saline containing 0.05% Tween 20) over night at 4°C cold room on a shaker to block non-specific binding sites. The next day the membrane was washed three times (15 min each) with 25 ml of TBS-T (For 1L TBS-T 8 g NaCl, 0.2 g KCl and 3 g of Tris base mixed and dissolve in 800 ml of milliQ water. pH was adjusted to 7.5, the volume was brought to 1L and 0.05% Tween 20 added) and then incubated with primary antibody (1:100-200 dilutions in 15 ml in blocking buffer) for one hour at room temperature under gentle shaking. The membrane was washed

three times with 25 ml of TBS-T buffer for 15 min each and incubated for one hour at room temperature with HRP-conjugated secondary antibody solution (1:7500 dilutions in 15 ml of blocking buffer) under gentle shaking covered with aluminum foil to avoid light. The membrane was then washed three times with 15 ml of TBS-T for 15 min each and finally incubated for 5-7 min in 1:1 mixture of luminol/enhancer and peroxide buffer (Immun-Star WesternC Chemiluminescent- BioRad or ECL Select Western Blotting Detection Reagent-GE Healthcare). 5 ml total solution (2.5 ml of luminol/enhancer and 2.5 ml of peroxide solution) was sufficient for one membrane. The developing solution was removed and the membrane was placed into a plastic wrap to prevent drying. An imaging system the Bio-Rad Molecular Imager ChemiDoc XRS was used to measure the intensities of obtained bands by applying Quantity One software. Tubulin was used as a loading control to make a comparison between the expressions of a protein in different samples (Fig.2, Fig.3, Fig.4, and Fig.5). Tubulin normalization was performed to be able to compare the expression levels (Fig.6 and Fig.7).

3.9 Western blots; primary and secondary antibodies

3.9.1 Primary antibodies

Table 2 Primary antibodies that have been used for both Western Blot and Immunohistochemical analyses

Protein	MW	Species	Conc. Dilution	Type	Source
BCRP/ABCG2 [BXP-53]	73.03 kDa	Reacts with an internal epitope of BCRP/ABCG2 in mouse and human	1:100 0.25mg/ml	Rat monoclonal	Abcam
Pgp (JSB-1 Antibody)	170.00 kDa	Anti-P-glycoprotein Mouse	1:200 1ml	Rat monoclonal	Abcam
Oatp3(Oatp1a5) (N-19):sc-47266	74.67 kDa	Epitope mapping near the N-terminus of Oatp3 of mouse origin	1:500 200µg/ml	Goat polyclonal IgG	Santa Cruz Biotechnology
Octn1 (P-12): sc-19820	64.00 kDa	Epitope mapping within an internal region of OCTN1 of mouse origin	1:200 200µg/ml	Goat polyclonal IgG	Santa Cruz Biotechnology
Anti-Tubulin antibody [YOL1/34]	50.00 kDa	Reacts with mouse, rat, dog and human	1:3000 100µg (1mg/ml)	Rat monoclonal	Abcam

3.9.2 Secondary antibodies

Table 3 Secondary antibodies that have been used for both Western Blot and Immunohistochemical analyses

Name	Conc. Dilution	Source
Donkey anti-goat IgG-HRP: sc-2020	1:7500 200 µg/0.5 ml	Santa Cruz Biotechnology
Rabbit Polyclonal Secondary Antibody to Mouse IgG-H&L (HRP) (ab6728)	1:7500 2 mg/ml	Abcam
Rabbit Polyclonal Secondary Antibody to Rat IgG-H&L (HRP)(ab6734) (Tubulin)	1:5000	Abcam
Polyclonal Goat Anti Mouse Immunoglobulins/Biotinylated (E0433)	1:200	Dako Cytomation

3.10 Stripping of nitrocellulose membranes

Stripping buffer contained 62.5 mM Tris-HCl, 100 mM 2-mercaptoethanol and 2% SDS was prepared to remove primary and secondary antibodies bound to a western blot membrane. All membranes were incubated 30 minutes at 60°C with 15 ml of stripping buffer on shaker. The membrane was washed three times with 25 ml of TBS-T buffer for 15 min each and incubated with suitable primary and secondary antibodies (for Tubulin primary antibody 1:3000 dilutions in 15 ml in blocking buffer and for Tubulin secondary antibody 1:7500 dilutions in 15 ml of blocking buffer were used).

3.11 Immunohistochemical detection of mouse Octn1

The lactating mouse mammary gland tissue was fixed for about 24 hours in 10% formalin to preserve the tissue. It was cut in suitable size and is placed in plastic cassettes. The plastic cassettes with the tissue were placed in a dehydration machine (histokinett). Tissue was dehydrated in alcohol from 70% to 100% and was treated in xylene and finally it was infiltrated in paraffin. The tissue was imbedded in paraffin in suitable forms to make a paraffin block. The paraffin block with the tissue was sectioned on a microtome in about 3 to 5 microns thin sections. They were placed on a glass slide and dried on a heating plate for about 1-2 hours.

The primary goat polyclonal antibody used for staining of Octn1 was prepared 1:50, 1:200, and 1:500 dilutions. The tissues were hydrated by a graded alcohol series starting with X-TRA Solv, 96% alcohol and continue with 70% alcohol. Antigen retrieval was performed with Tris/EDTA buffer containing 10mM Tris Base, 1mM EDTA solution and 0.05% Tween 20 at pH 9.0 at 98°C for 20 minutes. Then the tissues were blocked in two steps. First one was done by incubation in 1% hydrogen peroxide followed by a brief wash in PBS. 10% normal goat serum was then used for the second blocking step. The slides were incubated with the diluted primary antibody over night at 4°C and they were washed two times with PBS. 1:200 diluted secondary polyclonal goat anti mouse antibody was added for 30 minutes at room temperature. The tissues were washed with PBS several times and incubated with AB complex (Vestastain ABC kit) solution for 30 min derived from Vector lab which was prepared for half an hour before usage. Sections were washed again with PBS two times for 5 minutes for each followed by the addition of the DAB (3, 3'-diaminobenzidine) substrate solution and the reaction was stopped by immersing the slides in water. Sections were counter stained for 20 seconds in Mayers hematoxylin. The slides were washed and the tissues were dehydrated. After this the cover slips were mounted with X-TRA Kitt mounting medium and dried overnight.

4. RESULTS

4.1 BCA assay for determination of protein concentration by two different cell culture protocols

Two different cell culture protocols were used to induce differentiation using the same chemicals in HC11 cell line. As demonstrated in Table 4 a much higher protein concentration was achieved when we used second cell culture protocol. The result of the protein concentration from second protocol was almost 10 times higher than protein concentration from the first cell culture protocol in control HC11 cells. Protein concentration of differentiated HC11 cells was almost 40 times higher when in the second cell culture protocol.

Table 4 The protein concentration in HC11 cells from two cell culture protocols

Passage number 31	**First Cell Culture Protocol Protein Concentration (mg/ml)**	**Passage number 32**	**Second Cell Culture Protocol Protein Concentration (mg/ml)**
Control 1	0,61	Control 9	6,52
Control 2	0,67	Control 10	6,73
Differentiated 1	0,08	Differentiated 9	4,59
Differentiated 2	0,11	Differentiated 10	4,71

4.2 Western Blot analyses of transporter proteins expressed in HC11 cells

Protein expression of breast cancer resistance protein* Bcrp/Abcg2 *in HC11 cells and mouse mammary gland

Using Bcrp transporter specific antibodies applied to HC11 cells and mouse mammary gland the bands on 72 kDa corresponding to the predicted molecular weight of Bcrp were detected in Western Blots of HC11 cell line and positive control tissue samples. There was a remarkable upregulation of Bcrp in the mammary gland at lactating day 7 mouse compared to the virgin mice. Expression of Bcrp in HC11 cells looked similar based on Western Blot membrane images (Fig.2). As shown in fig. 6a based on protein/tubulin ratios Bcrp protein expression was almost two times higher in differentiated HC11 cells than in undifferentiated cells. This data agrees with the results shown in fig.7a that demonstrate an almost 15 times higher expression of Bcrp in mammary gland of lactating mouse than the virgin.

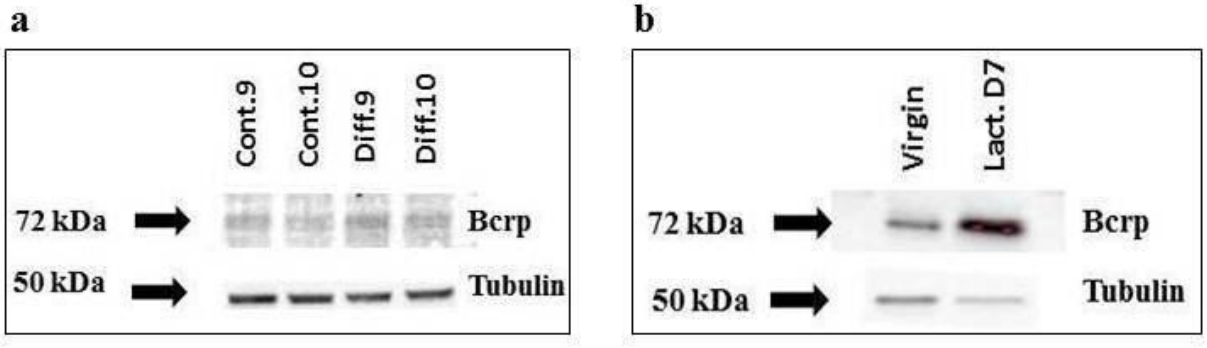

Figure 2 Protein expression of Bcrp/Abcg2 in the mammary gland. **(a)** Western blots on undifferentiated and differentiated HC11 cells (50µg per lane) with Bcrp antibodies and loading control tubulin. Two different controls (control 9 and control 10) and two different differentiated samples (differentiated 9 and differentiated 10). **(b)** Western blots of tissue samples from virgin and lactating day 7 female mouse mammary gland and loading control tubulin.

Protein expression of multidrug resistance protein* Mdr1/Pgp *in HC11 cells and mouse mammary gland

Transporter specific primary antibodies and corresponding secondary antibodies were applied to HC11 cells and mouse mammary gland epithelial cells. The detected bands were more closed to the molecular weight 250 kDa (Fig.3) in both in vitro and in vivo situations and it was hard to distinguish downregulation or upregulation on the blots without quantification analyses. After tubulin normalization Mdr1 tended to be somewhat downregulated in differentiated HC11 cells (Figure 6b). In lactating mouse mammary gland we found an increasing protein expression (Figure 7b).

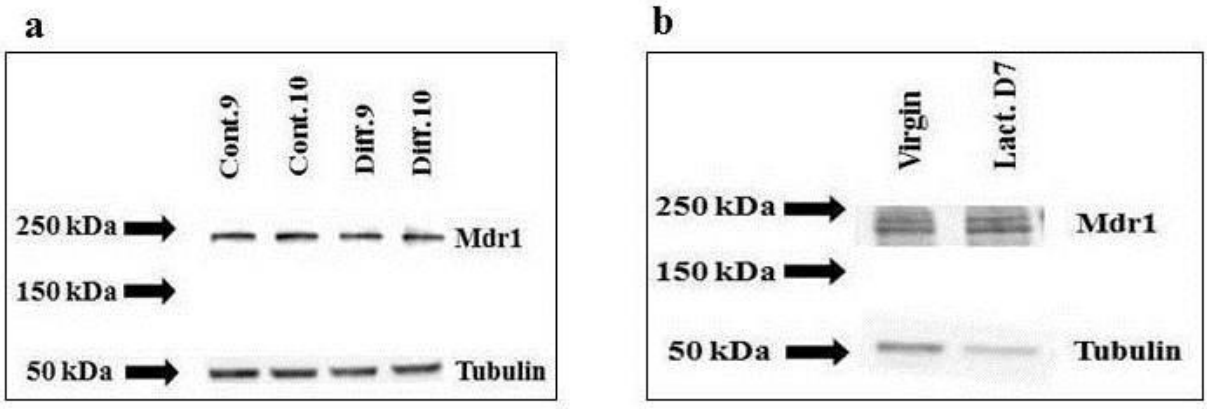

Figure 3 Protein expression of Mdr1/P-gp in the mammary gland. **(a)** Western blots on undifferentiated and differentiated HC11 cells (50µg per lane) with Mdr1antibodies and loading control tubulin. Two different controls (control 9 and control 10) and two different differentiated samples (differentiated 9 and differentiated 10). **(b)** Western blots of tissue samples from virgin and lactating day 7 female mouse mammary gland and loading control tubulin.

Protein expression of **Oatp3 (Oatp1a5)** *in HC11 cells and mouse mammary gland*

The protein expression of Oatp3 was examined using an antibody against N-terminus of Oatp3 of mouse origin in HC11 cells and also in mouse mammary gland epithelial cells. The immunoreactivity was detected in both HC11 cells and mouse mammary gland tissues at 75 kDa which indicates that the antibody reacts with mouse Oatp3 as expected (Fig.4a, 4b). Since the bands were weaker than expected, the comparison of the protein expressions between different samples has been done after tubulin normalization as shown in Figure 6 and Figure 7. The protein expression of this transporter was similar in both undifferentiated and differentiated HC11 cells as well as in tissue samples.

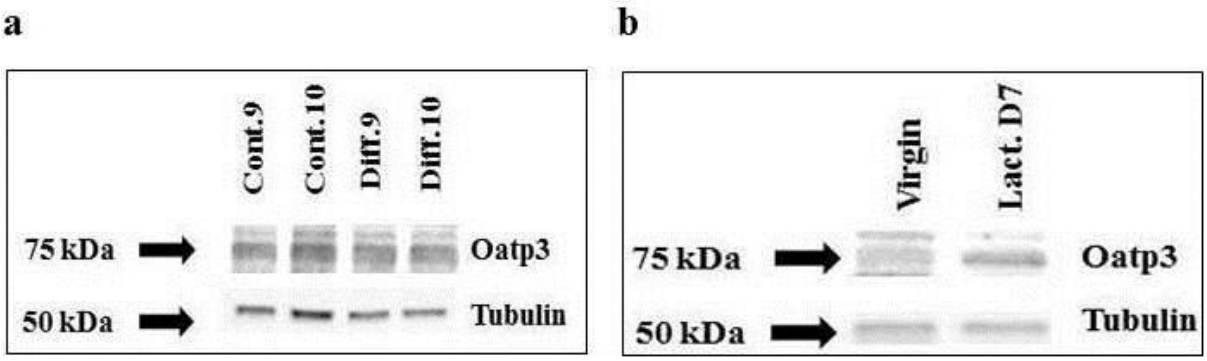

Figure 4 Protein expression of Oatp3 (Oatp1a5) in the mammary gland. **(a)** Western blots on undifferentiated and differentiated HC11 cells (50µg per lane) with Oatp3 antibodies and loading control tubulin. Two different controls (control 9 and control 10) and two different differentiated samples (differentiated 9 and differentiated 10). **(b)** Western blots of tissue samples from virgin and lactating day 7 female mouse mammary gland and loading control tubulin.

Protein expression of **Octn1** *in HC11 cells and mouse mammary gland*

The protein expression of Octn1 was examined using an antibody against the internal region of Octn1 of mouse origin in HC11 cells and also in mouse mammary gland epithelial cells. The bands were detected between 50 and 75 kDa since the predicted molecular weight of Octn1 is 64 kDa. The background was totally clear and only these bands appeared on the blot which indicates that the antibody specifically reacts with mouse Ocnt1 as expected (Fig. 5a, 5b). The comparison of the protein expressions between different samples has been done after tubulin normalization as shown in Figure 6 and Figure 7. Octn1 was upregulated in differentiated HC11 cells and also in mammary tissue from lactating mouse.

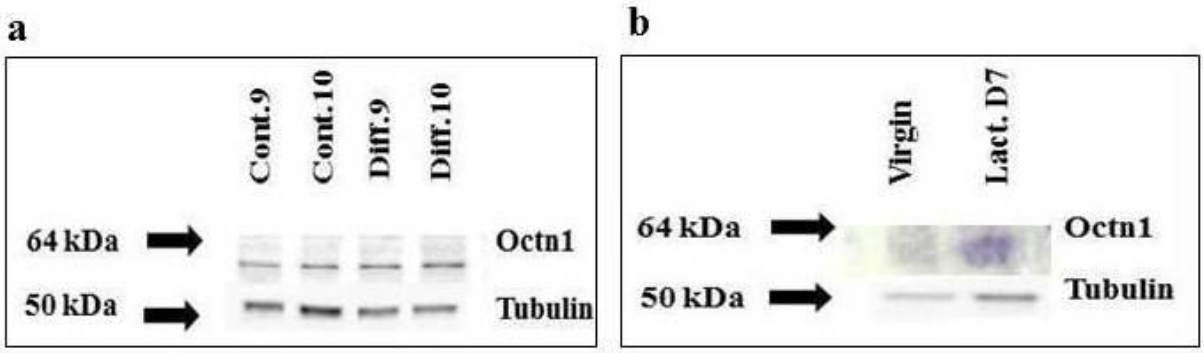

Figure 5 Protein expression of Octn1 in the mammary gland. **(a)** Western blots on undifferentiated and differentiated HC11 cells (50µg per lane) with Octn1 antibodies and loading control tubulin. Two different controls (control 9 and control 10) and two different differentiated samples (differentiated 9 and differentiated 10). **(b)** Western blots of tissue samples from virgin and lactating day 7 female mouse mammary gland and loading control tubulin.

For analyzing Bcrp, Mdr1, Oatp3 and Octn1 protein expressions in HC11 cells, experiments were repeated several times and the protein/tubulin ratios were calculated by taking average. Normalized expression levels of the transporters are illustrated in Figure 6.

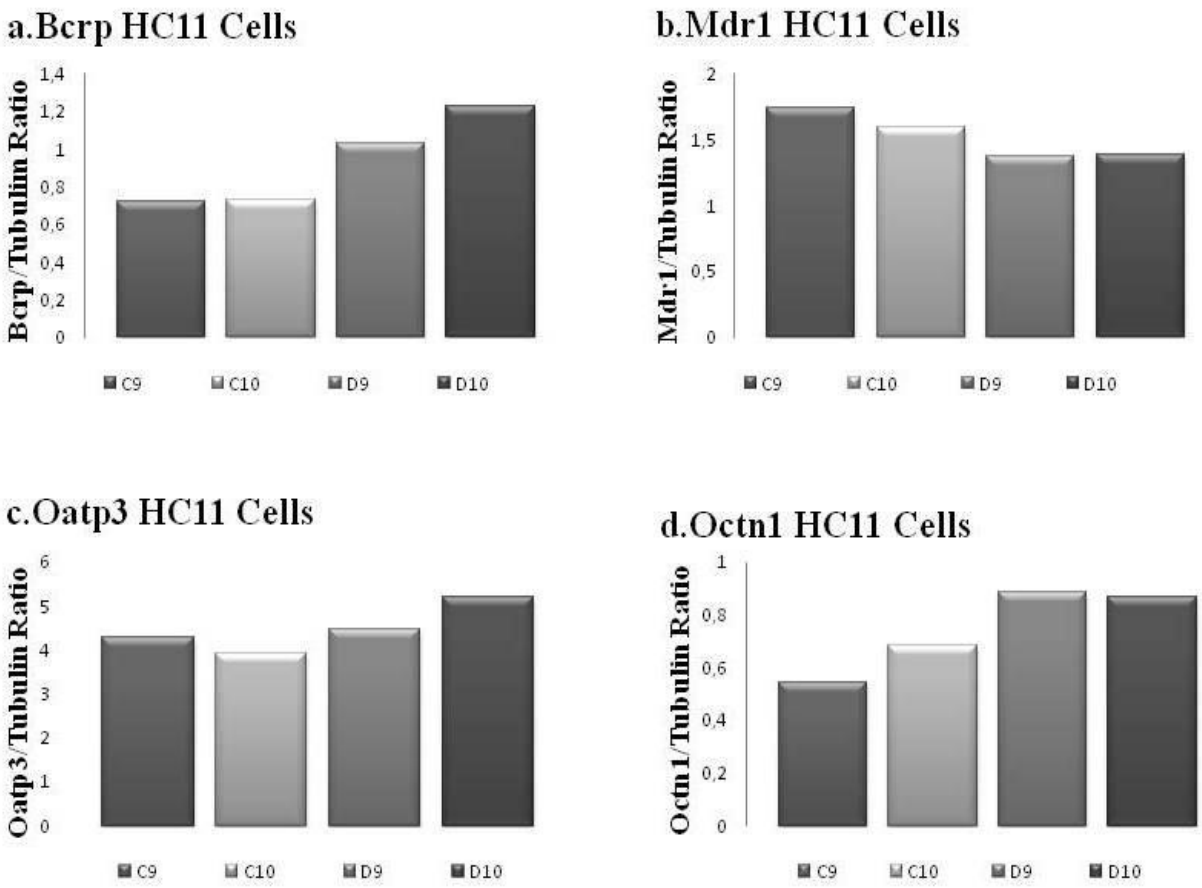

Figure 6 Quantification analyses of transporter proteins in HC11 cells by protein/tubulin ratio. Results from cells grown in individual culture flasks are shown. Two different controls (C9 and C10) and two different differentiated samples (D 9 and D10).

For analyzing Bcrp, Mdr1, Oatp3 and Octn1 protein expressions in tissue samples, experiments were repeated several times and the protein/tubulin ratios were calculated by taking average. Normalized expression levels of the transporters are illustrated in Figure 7.

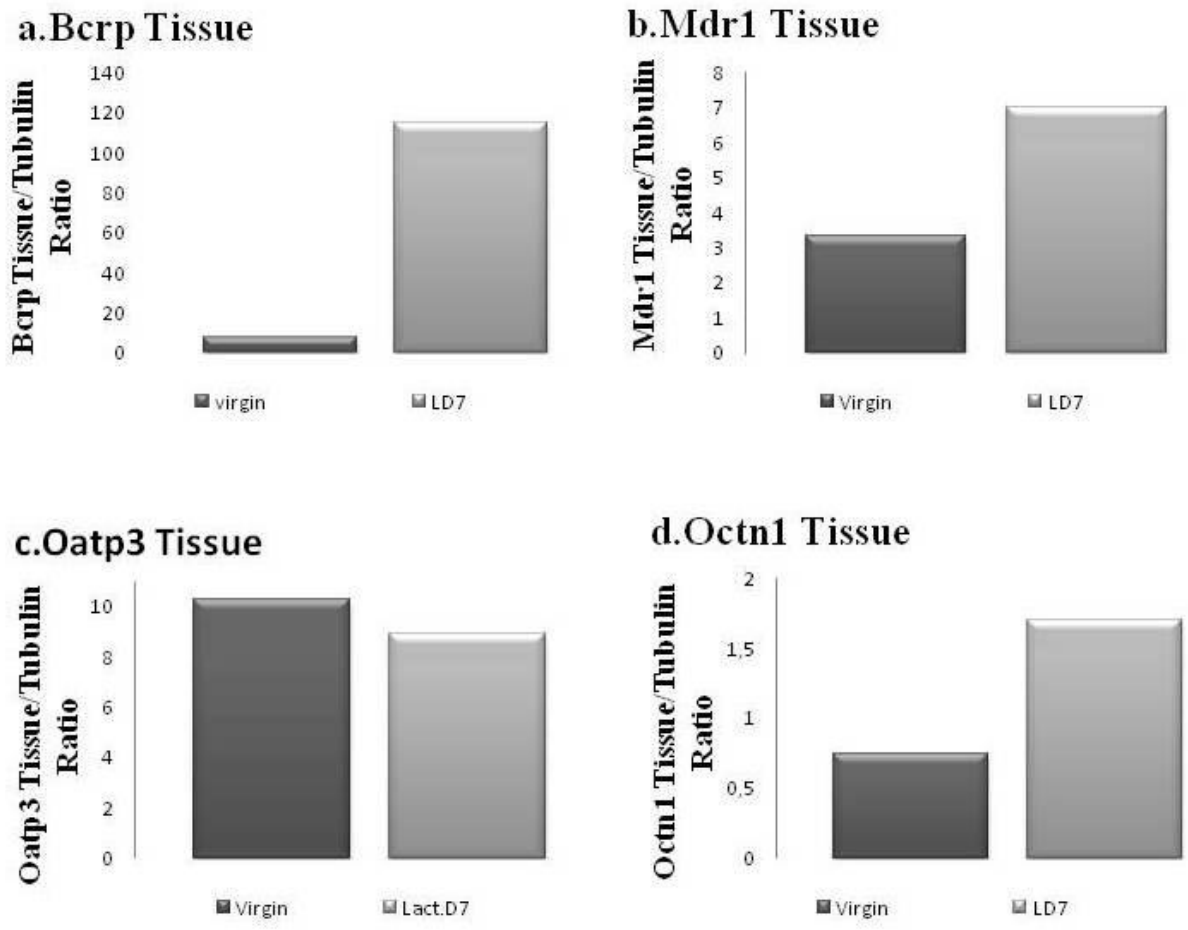

Figure 7 Quantification analyses of transporter proteins in mammary gland tissues by protein/tubulin ratio. Results from tissues obtained from individual mice are shown. Tissue samples from virgin and at lactating day 7 female mice mammary gland (LD7).

4.3 Western Blot analysis of LPS treatment

Protein expression* of Bcrp, Mdr1, Oatp3 *(Oatp1a5) and* Octn1 *in LPS treated HC11 cells

The protein expression of Bcrp, Mdr1, Oatp3 (Oatp1a5) and Octn1 was examined using specific antibodies to mouse origin to see if there is any differences at the protein expression levels when the differentiated HC11 cells were treated with lipopolysaccharides. The bands were detected at the predicted molecular weight for all transporter proteins (Fig.8). The comparison of the protein expression levels between different samples has been done after tubulin normalization as shown in Figure 9. We found similar values in both nontreated (NLPS) and treated (LPS) HC11 cells for all transporters (Figure 9).

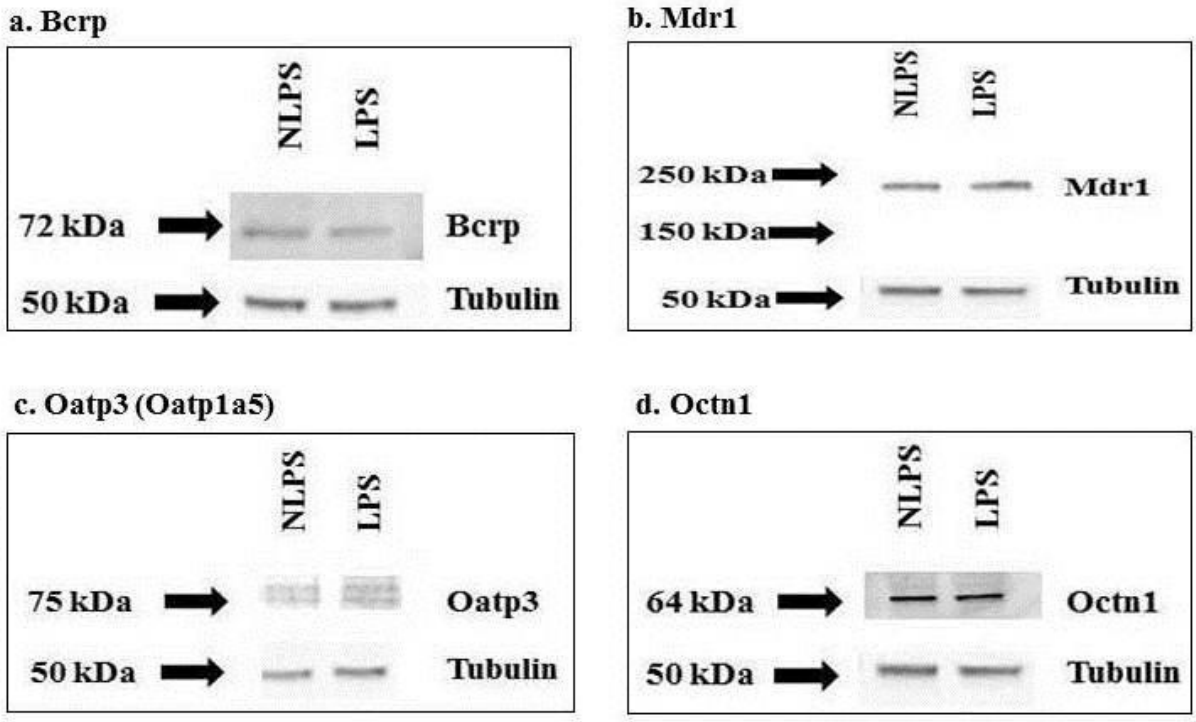

Figure 8 Protein expressions of Bcrp, Mdr1, Oatp3 and Octn1 in the LPS treated and non-treated (NLPS) HC11 cells (50µg per lane). **(a)** Western blots of mouse Bcrp on the LPS and NLPS treated HC11 cells and loading control tubulin. **(b)** Western blots of mouse Mdr1 on the LPS and NLPS treated HC11 cells and loading control tubulin. **(c)** Western blots of mouse Oatp3 (Oatp1a5) on the LPS and NLPS treated HC11 cells and loading control tubulin. **(d)** Western blots of mouse Octn1 on the LPS and NLPS treated HC11 cells and loading control tubulin.

For analyzing Bcrp, Mdr1, Oatp3 and Octn1 protein expression in LPS treated and untreated HC11 cell line, experiments were repeated several times only for some of the transporters because of the limited time and the protein/tubulin ratios were calculated by taking average to compare expression levels between different samples (Fig.9)

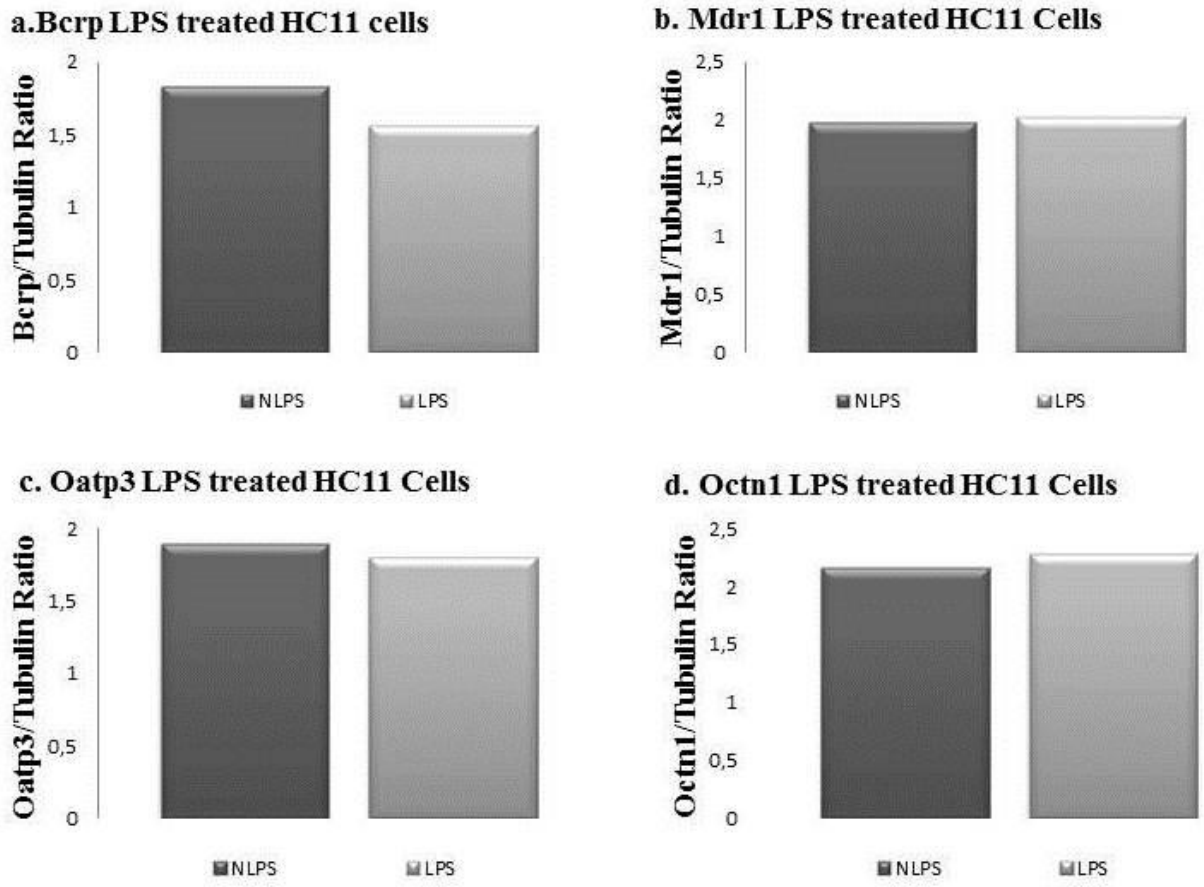

Figure 9 Quantification analyses of transporter proteins in LPS treated and non-treated (NLPS) HC11 cells by protein/tubulin ratio. Results from all secreting cells grown in individual culture flasks are shown.

4.4 LDH cell toxicity test

When the cells are treated by lipopolysaccharide (LPS) at cytotoxic levels, the enzyme lactate dehydrogenase (LDH) which is normally present in the cells will be released out of the cells in to the medium where the cells are growing. The concentration of LDH in the medium indicates the toxicity and the degree of damage of the cells. LDH toxicity test was performed to examine if there is any damage in the differentiated cells when they were treated with LPS. In this assay cell culture medium was used both from LPS treated and untreated cells. Two different positive controls were used containing low and high LDH, respectively to be able to analyze the cell damage. Duplicate samples were used for each sample and the mean value presented. Although it is unknown why untreated cell culture medium gave more LDH concentration than LPS treated cell culture medium, they were still lower than the sample which contains low LDH (Fig.10).

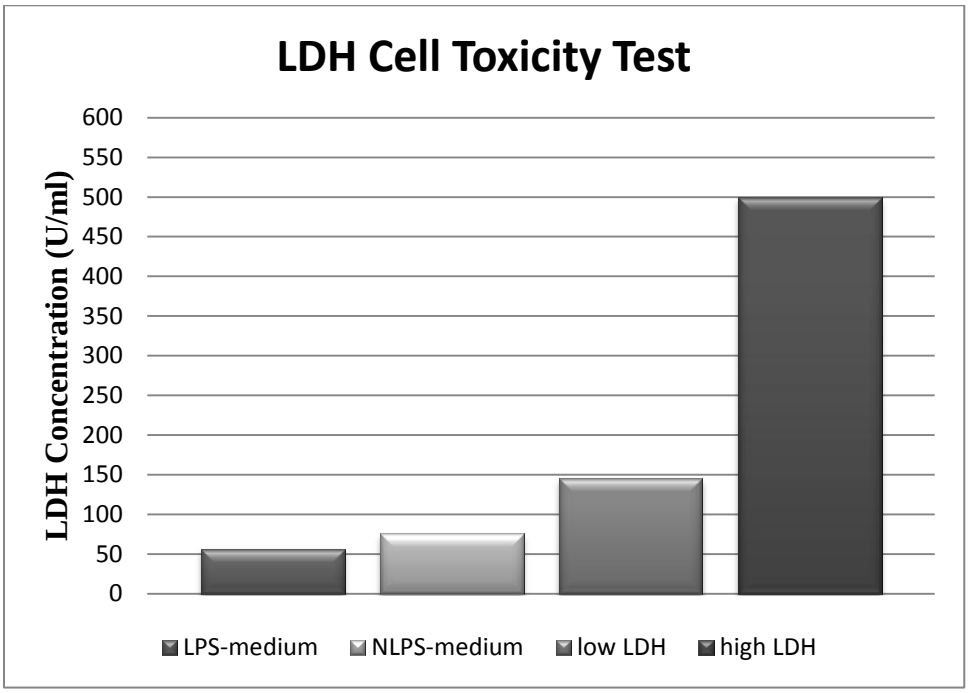

Figure 10 Results from cells grown in individual culture flasks are shown. LDH concentrations in one LPS treated cell culture medium and one non-treated (NLPS) cell culture medium. Two positive controls contain low and high LDH.

4.5 Immunohistochemical localization of Octn1 in lactating mammary glands of mice

Localization of mouse Octn1 in lactating mammary glands of mice was performed. The results showed that Octn1 protein is predominantly localized to the apical membranes of the mammary epithelial cells, whereas negligible staining was observed in myoepithelial and ductal cells (Figure 11 A). No staining was observed when primary antibodies were omitted and replaced by mouse specific IgG as negative control (Figure 11 B).

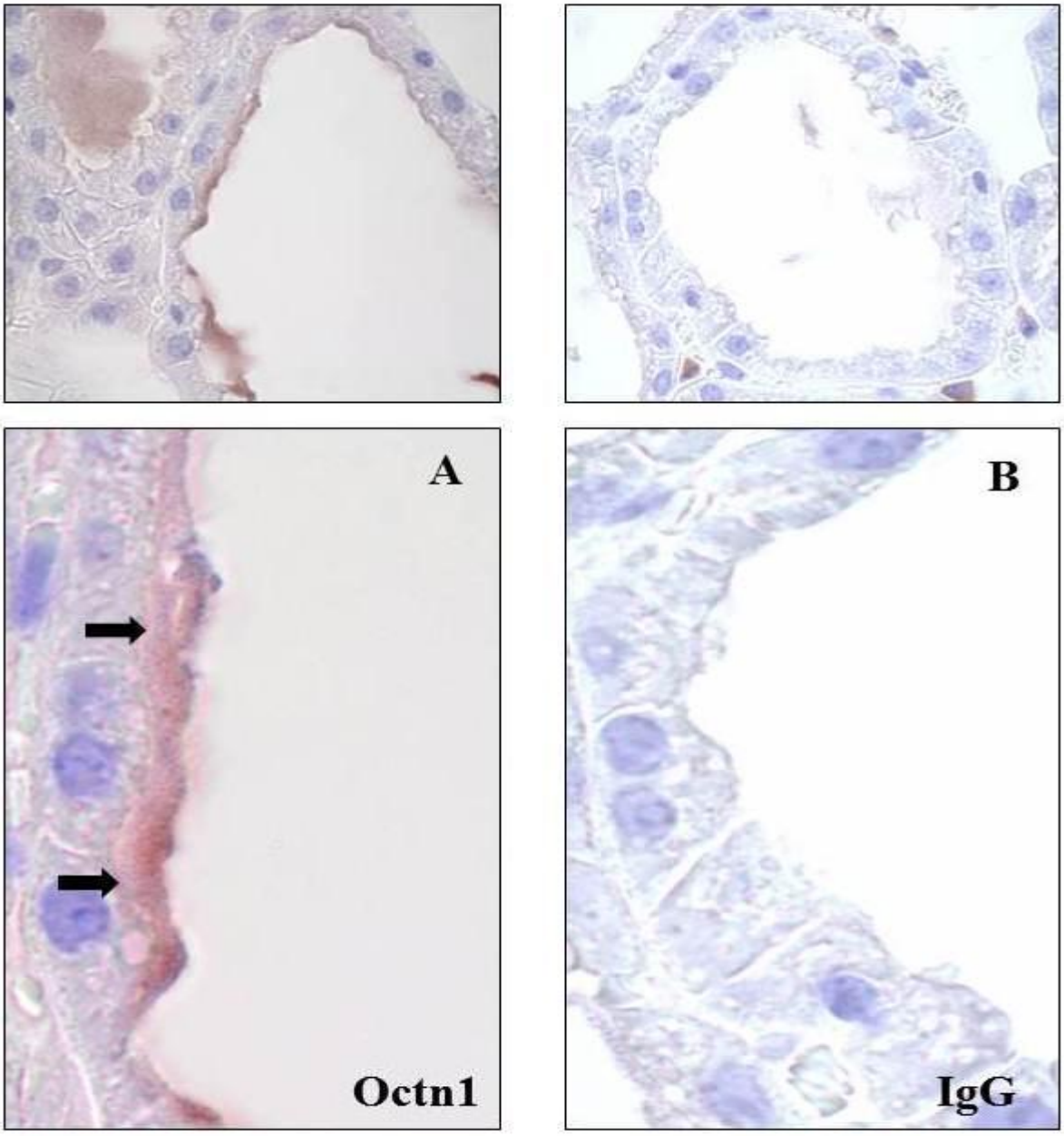

Figure 11 A localization of organic cation transporter Octn1 in lactating mammary gland epithelial cells of mouse by immunostaining (1000X magnification) on the upper two images. The mammary gland tissues were prepared as described in MATERIALS AND METHODS and 1:500 dilutions of the primary antibody are presented. The area that demonstrates the localization of Octn1was cropped and presented below these two figures. Arrows indicate localization of Octn1 protein as brown staining. **B** demonstrates a negative control of the epithelial cells stained with only negative control mouse IgG.

5. DISCUSSION

The biggest achievement of this study was to optimize protocols for Western Blotting of transporter proteins isolated from murine HC11 mammary epithelial cells. The optimal protocol was developed and used to investigate protein expression of transporters in secreting mammary epithelial cells. Since this method contains many steps, we have faced a lot of problems such as high background, no signaling, multiple bands, white spots or black dots on the blot etc. until the protocol was optimized. Although most of the problems seemed to be solved and the method is a promising tool, it still needs to be repeated to obtain best results.

Transporter proteins are involved in active transport of different exogenous compounds and substances into milk. In the present study we have examined protein expression of mouse Bcrp, Mdr1, Oatp3 (Oatp1a5) and Octn1 in HC11 cells. As a quality control protein expression of the transporters was also analyzed in mouse mammary tissue from a virgin mouse and from a mouse on lactation day 7. The undifferentiated control cells were used as model of non-secreting mammary gland epithelial cells while the differentiated cells were used as model to mammary gland epithelial cells of lactating mouse. As shown in Figure 2 the expected band size 72 kDa was achieved in the presence of 50 μg proteins for mouse Bcrp in both HC11 cells and tissue samples. Bcrp expression in mammary glands of lactating and nonlactating mouse correlated with the results reported by Jonker et al., 2005 in mouse. In the present study Bcrp expression was high and seemed to be upregulated in the mammary gland of lactating mouse as well as in the differentiated HC11 cells (Figure 6a and Figure 7a). As shown in the fig. 6a based on protein/tubulin ratios Bcrp protein expression was almost two times higher in differentiated HC11 cells than undifferentiated cells. This data agrees with the results shown in fig.7a that represent almost 15 times higher expression in mammary gland of lactating mouse than the virgin.

We have shown that mouse Mdr1 is expressed both in HC11 cells and mammary gland tissue samples. Mdr1 has high molecular weight with 170 kDa and the detected bands of interest were more closed to the higher molecular weight 250 kDa on the blot (Figure 3). Since this protein is quite big, the separation of the proteins might not be efficient due to the relatively high gel percentage or it may need to be incubated more than 5 minutes at 95°C to assure all the disulfide bonds were cleaved. We have also detected multiple bands for tissue samples from virgin and lactating day 7 mouse mammary glands. Also some novel proteins or different splice variants that share similar epitopes and could be possibly from the same protein family might have been detected. Tubulin detection on the predicted size was efficient

even though it was weaker for tissue samples than the detected bands for HC11 cells (Figure 3b). After tubulin normalization Mdr1 tended to be somewhat downregulated in HC11 cells (Figure 6b) in differentiated cells. Furthermore, although it has been shown by Jonker et al., 2005 that Mdr1 was downregulated during lactation in mouse with a very strong decreased protein expression, we have demonstrated totally opposite results with an increasing protein expression in lactating mouse mammary gland (Figure 7b). According to these preliminary results, the experiment should be repeated.

Mouse Oatp3 (Oatp1a5) protein expression was detected in both HC11 cells and tissue samples demonstrating that the specific antibodies reacted with mouse Oatp3 at the predicted molecular weight 75kDa as expected (Figure 4a and 4b). After tubulin normalization the data gave similar values in both undifferentiated and differentiated HC11 cells as well as in tissue samples. Upregulation of OATPA (ortholog of mouse Oatp3) at gene level has been reported by Alcorn et al., (2002) in lactating human mammary epithelial cells. But in the present study there were no effects of mouse Oatp3 neither in HC11 cells nor mouse mammary gland tissue samples (Figure 6c and Figure 7c).

As shown in Figure 5a, b expected bands were detected between 50 and 75 kDa for Octn1. Although the estimated size of Octn1 is ~ 64kDa, the detected bands were consistently closer to 50 kDa in several trials, suggesting post transcriptional modifications or degradation. Octn1/Tubulin ratio data showed that the organic cation transporter Octn1 was upregulated in differentiated HC11 cells and also in tissue samples from lactating mouse (Figure 6d and Figure 7d).

Our present study showed expression and localization of Octn1 by immunohistochemistry staining using polyclonal goat antibody against mouse Octn1. Staining seemed to be very specific as shown in Fig. 11A since the dilution factor of antibody was high (1:500). Method should be optimized to improve these preliminary results by decreasing the dilution factors or trying different blocking kits. Octn1 is predominantly located at the apical membrane of lactating mouse mammary epithelial cells. It sounds logical when we think about carnitine delivery to the new born through breast milk suggesting that Octn1 can be an efflux transporter since carnitine has to be transported actively into the milk to improve early brain growth and development of the newborn.

Another aim of this project was to investigate impacts of lipopolysaccharide (LPS) inflammation on protein expression of transporters in secreting HC11 cells. The

immunoreactive bands for four different transporters were detected on the estimated sizes on western blots (Figure 8). It is believed that inflammation can alter the expression of membrane transporters at both gene and protein levels. It has been reported by Ling et al., (2010) that mRNA expression levels of glucose, fatty acid and carnitine transporters in whole mammary tissue treated with LPS are dependent on different lactation stages and these authors demonstrated that LPS administration markedly increased Octn1 expression. The expression alterations by inflammation in both gene and protein levels have also been reported for one of the ABC family efflux transporters Mdr1 by Al-bataineh et al., (2010). According to RT-PCR and Western Blot analyses they demonstrated a significant increase in the gene and protein expression levels of Mdr1 after inflammation. However, in the present study we found similar values in both nontreated (NLPS) and treated (LPS) HC11 cells for all transporters (Figure 9). When the LDH cell toxicity test was performed to detect membrane damage and asses cell toxicity, it was also concluded that the used concentration of LPS was not toxic. It might be argued that the absence of effects could be due to a too low concentration of LPS. It could also be different sensitivity in different cell lines and by different inducers of inflammation.

HC11 *in vitro* cell model was used in all these experiments. Two different cell differentiation protocols were used to optimize the experimental conditions. In this study we were able to achieve a much higher protein concentration when we used second cell culture protocol. As presented in Table 4, when the second culture protocol was performed on HC11 cell differentiation, the result of the protein concentration was almost five times higher than the first cell culture protocol protein concentration. Although different incubation times was performed in these two protocol to induce differentiation, the difference of protein concentrations was probably caused by some steps during protein extraction including detachment methods, using lower volumes of lysis buffer or using different size of cell culture flasks.

6. CONCLUSION

In this cell model it was possible to detect upregulation of membrane transporters mouse Bcrp and Octn1 at differentiation stage. Based on the observed results, further investigations on membrane transporters expressed in the mammary gland and the active secterion of nutrients into the milk by these transporters are definitely needed. Octn1 was localized at the apical membrane of mammary epithelial cells which is in line with its role in the transport of carnitine into milk. More studies are required to understand the possible role of Octn1 in transporting xenobiotics in the mammary epithelium. No effect was detected by LPS on protein expression of transporters in secreting mammary epithelial cells possibly because of a too low LPS concentration. The latter findings should be clarified by exposing the cells for various LPS concentrations and/or longer periods to elucidate effects of inflammation on gene and protein expression.

7. ACKNOWLEDGEMENTS

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