Gene expression of transporter proteins in murine mammary epithelial HC11 cells

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Abstract

Milk is primary food of infants and indispensable part of human diet. There is solid evidence of chemicals and drugs presence in milk which raise concerns for toxicological safety of milk. Membrane transporters play key role in the transport of chemicals into milk. Transporters belong to ABC transporter superfamily e.g. Bcrp, Mdr1 and Mrp1 or SLC transporter family e.g. Oatp3 and Octn1. Another considered issue is mastitis which is inflammation of mammary gland usually due to bacterial infection. Two major causes of mastitis are *Staphylococcus aureus* and *Escherichia coli.*

In this project we studied gene expression of several ABC transporters; *Bcrp1*, *Mdr1*, *Mrp1* and SLC transporters *Oatp3* and *Octn1* in murine mammary epithelial HC11 cells differentiated into secreting phenotype. We compared two different cell differentiation protocols on HC11 cells and their potential effect on gene expression of membrane transporters. We also investigated effects of *S. aureus* infection and endotoxin treatment (LPS) on gene expression of transporters. Expression of transporters was studied by SYBR Green RT-PCR.

The results showed that both cell differentiation protocols caused a downregulation of *Bcrp* and *Octn1*. In addition, cell differentiation protocol I resulted in a downregulation of *Mrp1* and upregulation of *Oatp3*. Furthermore, cell differentiation protocol II resulted in a downregulation of *Mdr1*. *Octn1* was upregulated in presence of *S. aureus* and *Oatp3* by LPS treatment.

In conclusion, HC11 cells were demonstrated to express all the investigated membrane transporters. Some differences between the two evaluated differentiation protocols were detected, regarding gene expression of the membrane transporters. However, a common observation for both differentiation protocols was a downregulation of Bcrp and Octn1. Bacterial infection of HC11 cells resulted in upregulation of *Octn1*, whereas LPS treatment induced upregulation of *Oatp3*. Further studies are needed to elucidate the physiological role of reduced *Bcrp* and *Octn1* in secreting HC11 cells and also to confirm the upregulation of *Octn1* in *S. aureus* infected cells and *Oatp3* in LPS treated cells

Key words: milk, HC11 cells, *Bcrp*, *Mdr1*, *Mrp1*, *Oatp3*, *Octn1*, mastitis

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1. Introduction

1.1. Mammary gland and milk production

Milk is an important source of nutrients for mammals. It is highly recommended to include it in daily diet, and WHO recommends mothers to breast feed their newborns for at least 6 months due to mothers' milk high nutritional quality and reinforcement of immunity system of babies. Milk consumption continues to increase globally in response to the growing demand every year, e.g. there has been 1.4% increase in its production in Sweden from February of 2011 till February of 2012 (1). There has been solid evidence of chemicals and drugs presence in milk which raises concerns for toxicological safety of milk. Based on extensive studies conducted over transfer of drugs into milk, WHO and American Academy of Pediatrics enlist available drugs in safe, unsafe or with unclear effects categories for breast feeding women use (2, 3).

1.1.1. Mammary gland development

Mammary gland development begins at embryonic stage and continues till parturition. At puberty, secretion of estrous hormones commences development of mammary gland by triggering epithelial cells proliferation which eventually leads to further development of primary ductal structure. In the next step, alveolar buds are formed but they require pregnancy for terminal differentiation (4).Secretion of progesterone and prolactin during late pregnancy are needed for terminal differentiation of mammary gland and appearance of alveoli clusters arranged as secretory lobules, which ultimately produce and secret milk (5, 6). β-casein expression and formation of mammospheres are two typical markers of terminal differentiation of mammary cells and their full competence for secretion of milk *in vivo* (7).

1.1.2. Milk proteins expression and hormonal regulation

Initiation of milk protein expression is similar in humans and mice. Major milk proteins are expressed at negligible levels in virgin mice. During gestation, some of the milk protein genes start to be expressed while expressions of other genes are mainly increased after onset of lactation. At gestation, β-casein appears as the first milk protein, followed by whey acidic protein (WAP) and α-lactalbumin at late gestation (6, 8).

Hormones play key roles in regulation of cellular differentiation of mammary gland. Generally Epidermal Growth Factor (EGF), estrogen (secreted from ovary in estrous cycles), and glucocorticoids are required for early development of mammary gland (ductal formation) (6, 9).

Alveolar buds differentiation is prompted by prolactin and progesterone during pregnancy. Progesterone arrests β-casein expression during gestation but its level decreases at proximity of parturition, while glucocorticoids and prolactin levels commence to rise. Prolactin level augmentation leads to terminal differentiation of mammary gland, stimulation of β-casein expression (β-casein gene has regulatory elements interacting with Stat5, a signaling factor which transfers prolactin response (8)) and closure of tight junctions between alveolar cells.

Glucocorticoids are required for lactation onset, to induce both milk proteins and lipid production. Furthermore, tight junction formation is highly dependent on these corticosteroids (9, 10).

1.1.3. Secretion pathways in mammary gland cells

Two prominent pathways of chemical compounds entrance into mammary lumen are transfer by passive diffusion and membrane embedded transporters.

Passive diffusion connects interstitial space and lumen to each other. This route is straight, two-way, cell-independent, and always available even when tight junctions are present, but secretion of chemicals is highly dependent of several characteristics (11). Molecular size (molecules with 200 D can pass membrane pores), lipophilicity, ionization degree, protein-binding affinity and half-life in blood determine their passive diffusion into milk (12).

Membrane transport pathway briefly consists of several types of routes each governed by some kinds of proteins. Ion secretion is conducted by channels in apical membrane, glucose is transported by especial transporters in apical and basolateral membrane, and amino-acids are secreted through sodium dependent and independent transport mechanisms. Also xenobiotics can be transported from blood first across the basolateral membrane of mammary epithelial cells then across the apical membrane of these cells into milk by transporters (11).

1.2. Transporters in mammary gland

Two distinctive groups of membrane transporters have been found in charge of drugs and xenobiotics transfer across cellular membrane. First group is called ATP-binding cassette (ABC), responsible for energy dependent excretion of chemicals. This is a large super family of transporters with a similar primary structure and function. All members have two distinctive structural domains, transmembrane and nucleotide binding domains (NBD). NBDs provide the energy for chemical transport by hydrolyzation of ATP (13, 14).

The other prominent group of transporters is Solute Linked Carrier family (SLC) consisting of 43 super families of proteins in humans. SLC family members are involved in sodium independent transfer of chemicals across cellular membrane (13). In contrast to ABC transporters which actively transport chemicals only in one direction, SLC members can transfer chemicals in two directions and they do not utilize ATP energy for their activity (14).

1.3. Breast Cancer Resistance Protein (*Bcrp*)

Breast cancer resistance protein (Bcrp) is an ABC transporter discovered in 1998 by Doyle et al., in MC7/AdrVp- a type of multidrug resistant breast cancer cell line (15). It is encoded by *Abcg2* (*Mus musculus*) and belongs to ATP- binding cassette, subfamily G, member 2. *Abcg2* encodes a 657 amino acids polypeptide and 10 splice variants (16). Structural studies confirmed that it has half of the ABC transporter structure and it should dimerize prior to activation (17).

BCRP can pump out mitoxantrone, methotrexate, and topotecan and so many other chemicals and xenobiotics which makes it very crucial for homeostasis and cellular protection against chemicals (18).

BCRP is widely present in different tissues like apical membrane of placenta syncytiotrophoblasts, bile canalicular membrane of liver cells, luminal membrane of intestinal epithelial cells, capillary endothelial cells of most tissues apical side of alveolar epithelial cells' membrane in mouse, human and cow (18, 19).

Immunohistochemical and western blotting experiments showed that it is not expressed in virgin mammary tissues but it is extensively expressed through end of pregnancy and throughout lactation (18).

1.4. Multidrug Resistance Protein 1 (*Mdr1*)

Multidrug Resistance Protein 1 (Mdr1) also known as P-gp is a glycoprotein belongs to ABC transporters family, subfamily of B (or MDR/TAP) member A1 (*Abcb1a*, *Mus musculus*). It was discovered originally in a Chinese hamster ovary by Juliano et al., in 1976 (21). MDR/TAP family is known to confer cancer resistance, due to export of chemotherapeutics out of cells. Mdr1 activity in cells renders multi-drug cancer resistance as a result of chemicals removal from cells (20).

Studies on human ortholog of Mdr1 revealed that is usually transcribed from exon 1 where its promoter located with no TATA box (22). Its upregulation seems to be provoked by NF-κB induction, increase of cytosolic concentration of Ca^{+2}, and protein kinase C and mitogen- activated protein kinases cascades (23) While, its downregulation is due to epigenetical changes and methylation (24).

Generally it transfers neutral or very slightly positively charged lipophilic compounds (25) like vinca alkaloids, anthracyclines, and actionomycine D (20). Studies in humans and rodents showed that it is expressed in almost all tissue but its extensive expression can be detected in tissues with secretory activity e.g. apical membrane of colon, small intestine, kidney and adrenal gland. It is also a crucial component of blood-brain barrier. In mammary tissue it has been located in apical membrane of epithelial cells and its expression seems to be down-regulated in comparison to pregnancy in both humans and rats (26, 27).

1.5. Multidrug Resistance Associated Protein 1 (*Mrp1*)

Multidrug Resistance associated protein 1 (*Mrp1*) was first discovered by Cole et al., in 1992 in a drug resistant lung cancer cell line (28). It belongs to ABC family of membrane transporters, subfamily C (CFTR/MRP), member1 (*Abcc1 Mus Musculus*). It transports a variety of endogenous molecules, such as glutathione- conjugated leukotrienes and prostaglandins. It also protects cells against xenobiotics or internal toxins (steroids) by pumping them out (20). Mrp1 and Mdr1 display high similarity in their activity, it also excretes vinca alkaloids, and actinomycin D like Mdr1. Yet Mrp1 tends to transfer lipophilic compounds with negative charge (25). It can transfer high concentrations of methotrexate, and especially transfer glucoronate, sulfate and gluthathione conjugated substrates (20).

Its tissue distribution in human and mouse differs. Human MRP1 is abundantly found in lung, spleen, bladder, and thyroid but mouse Mrp1 is highly available in ovaries, placenta and testes (25).

In a study by Alcorn et al., *Mrp1* expression was studied in rat whole mammary gland derived from different days of pregnancy and lactation. Its expression was downregulated by onset of lactation and this trend lasted till end of lactation while it was upregulated in early involution (26). Its downregulation is also reported in another study of Alcorn et al., in mammary epithelial cells of lactating rat (27).

1.6. Organic Anion Transporting Polypeptide 3 (*Oatp3*)

Mouse Organic Anion Transporting Polypeptide 3 (OATP3) was discovered by Abe et al., in rat retina's cDNA library (29). It is encoded by solute carrier organic anion transporter, member 1 a 5 (*Slco1a5*), with seven transcripts variants.

It has been detected as an influx transporter in transfected COS cells with sodium independent activity. It has been also identified in apical side of MDCK cells and small intestine jejunal probably for uptake of bile acid (30).

Xenopus Oocytes containing *Oatp3* cRNA were capable of absorbing taurocholate, 3,5,3'-triido-L-thyronine,and thyroxine (29). In addition, MDCK cells Transfected by rat *Oatp3* could absorb glycocholate, glycodeoxycholate, and taurodeoxycholate (30).

It has been localized in the brain (chroiod plexus), and epithelial cells of saccule (ear) of mouse and lung, retina and small intestine of rat (30).

Its expression has not been studied in rodents but its human ortholog, OATP- A encoded by gene (*SLCO1a22*) (30) displayed upregulation in lactating mammary epithelial cells in comparison to non-lactating epithelial cells (27).

1.7. Organic Cation Transporter, Novel Protein, Type1 (*Octn1*)

Organic Cation Transporter, Novel Protein, Type1 (Octn1) encoded by the SLC family 22, member a4 (*Slc22a4*, *Mus musculus*) was first discovered by Tamai et al., (1997) as a pH dependent transporter of positively charged organic compounds in humans (31).

In addition to its well-studied substrate $_{L}$-carnitine, it transfers other positively charged compounds like, tetra-ethylammonium (TEA), pyrilamine, verapamil (33).

It has been localized in human fetal and mouse liver, and apical side of epithelial membrane of mouse small intestine (33). It is extensively found throughout mammary

gland, localized at apical membrane and to less extent in basolateral side of secretory alveolar cells in rat and mouse, it has been shown that Octn1 is present in organelle's membrane e.g. mitochondria (32, 34).

Its expression in mammary gland has been subject of several studies, both Alcorn et al., and Lamhonwah et al., reported its upregulation in lactating mammary gland in comparison to non-lactating mice and human (27, 34). In another study by Alcorn et al., its expression in rat whole mammary gland was studied and it displayed continuous upregulation from parturition till day 3, but it decreased slowly through end of lactation (26).

1.8. Mastitis

Mastitis is the inflammation of mammary gland usually due to bacterial infection within it. Mastitis is the major cause of expense among dairy cattle diseases especially due to reduction of milk production in infected animals leading to staggering financial losses. *Staphylococcus aureus* is the most common cause of mastitis (35, 36). Pathogens enter through teat canal and if they endure animal defense system and proliferate, tissue damage occurs including destruction of alveolar cells and disruption of blood-milk barrier (35, 37).

Another common cause of mastitis is *Escherichia coli,* which may invade animal in parturition proximity or early stages of lactation. It also causes severe tissue damage and decrease of milk production. *E.coli* releases endotoxin after its death which is called lipopolysaccharide (LPS) (38).

1.9.HC11 cell model

HC11 cell line is a clonal derivation of COMMA-1D mouse mammary epithelial cell line first established by Danielson et al., in 1984. COMMA-1D cell line is isolated from murine mammary tissue of BALB / c during mid-pregnancy and quite stable even after 15 subcultures. It maintains main characterization of murine mammary epithelial cells. They can gain and retain lactating phenotype after exposure to certain amount of hormones and growth factors (39).

HC11 cells proliferation requires EGF and insulin, EGF stimulates formation of lactogenic hormone responsive cells needed for differentiation (40).

Cells differentiation takes place by addition of prolactin, glucocorticoid and insulin, while EGF should be removed from medium in prior. Differentiation can be confirmed by obtaining spherical phenotype, β-casein expression upregulation and apical to basolateral polarization necessary for secretion of milk proteins through lactation. About 60 proteins have been identified to be upregulated in differentiated cells e.g., cellular components and cytoskeleton proteins undergo upregulation resulting in differentiated phenotype in HC11 cell line (41).

2. Aims of Study

The primary aim of this study was to examine the occurrence of several membrane transporters at the gene expression level; *Bcrp*, *Mdr1*, *Mrp1*, *Oatp3* and *Octn1* in murine mammary epithelial HC11 cells differentiated into a secreting phenotype. Other aims were to compare the impact on membrane transporter gene expression in secreting HC11 cells stimulated to differentiate by two different protocols and the effects by *S.aureus* infection and LPS treatment.

3. Materials and Methods

3.1. Cell culture

The HC11 mouse mammary epithelial cells used in the experiments were a kind gift of Dr. Bo Lönnerdal, Department of Nutrition, University of California, Davis and used with the permission of Dr. Bernd Groner, Institute of Biomedical Research, Frankfurt . Passage numbers 26-36 were used in the experiments. The HC11 cells were cultured in sterile Roswell Park Memorial Institute (RPMI) 1640 basal medium , pH 7.4, (2mM L-Glutamine ,25 mM HEPES) (GIBCO, Invitrogen, Carlsbad, CA, USA), Gentamycin 50 mg/L (Gibco, Invitrogen, Carlsbad, CA, USA) , Sodium Bicarbonate 7.5% (w/v), human insulin solution 5mg/L (Sigma- Aldrich, St. Louis, USA), Epidermal growth factor (EGF) 10 µg/L, and 10% (v/v) heat –inactivated -Foetal bovine serum (FBS) (Gibco, Invitrogen, Carlsbad, CA, USA). The cells were cultured at 37˚C and 5% CO_2 in a Sanyo incubator (MCO-18AIC (UV), Japan). Medium was changed every second day.

To passage cells, when they reached 80% confluence , the growth medium was removed and cells were washed by DMEM (15ml/75 cm^2) and 3 ml of cell dissociation buffer

(enzyme-free PBS-based) (Gibco , Invitrogen , Paisley , UK) was added to the cells. Cells were then incubated for 20 minutes in incubator at 37 ˚C . Thereafter, 12 mL of growth medium added to the cell culture flask and cell suspension prepared by pipetting up/ down several times. Centrifugation of the cell suspension was then performed for 5 minutes at 50x g . Prior to centrifugation a 160 µl aliquot was taken from the cell suspension which was stained by addition of Trypan blue 0.4% (Gibco, Invitrogen). Counting of cells was then carried out by the use of a Bürchner chamber. After this, the cell pellet was resuspended in growth medium. For seeding cells in 6 well- plate 10^5 cells were added to each well and for T75 flask $1.5x10^6$ cells were added.

3.1.1. Cell differentiation protocol I

Cells were cultured in growth medium 4 days post 100 % confluency. The cells were then washed with DMEM (15ml/75 cm^2 or 2 ml/ per well for 6 well-plate) in order to remove medium residues and dead cells. DMEM was removed and replaced with differentiating medium. Differentiating medium had a similar composition as the growth medium with the difference that FBS and EGF were replaced by prolactin 1 mg/L (Sigma- Aldrich, St. Louis, USA) and cortisone 1 µM (Sigma- Aldrich, St. Louis, USA). Stimulation of the HC11 cells to differentiate into a secreting phenotype was accomplished by incubating the HC11 in differentiating medium for 3 days.

3.1.2. Cell differentiation protocol II

When cells reached 100% confluency, they were cultured in growth medium for an additional 6 days. On day seventh of postconfluence growth medium was removed and cells were washed by DMEM (15ml/75 cm^2 or 2 ml/ per well for 6 well-plate) to thoroughly remove all EGF and then cultured in EGF-free growth medium for one day. Cells were washed with DMEM as described above and then incubated with differentiating medium for at least 3 days. Lactogenic phenotype- mammospheres were observed and checked by phase-contrast microscope Olympus as an indication of true cell differentiation.

3.1.3. Induction of inflammation by *Staphylococcus aureus*

Cells were differentiated according to cell differentiation protocol II in two six well plates. One day prior to the infection experiments cells were incubated with antibiotic-free differentiating medium. On each plate 1x 10ˆ 6 colony forming unit (CFU)/ml was added to three of the wells while only medium was added to other three wells (used as negative controls). The total volume added to each well was 3 ml. Cells were incubated at 37 ˚C for two hours, medium was then removed and cells were washed by HBSS twice. After washes, RPMI medium containing 100 µg/ml gentamycin was added and plates incubated for an additional five hours. After this, cells were washed with HBSS and either lysed with RA1 buffer (Macherey- Nagel) for RNA isolation or Milli Q water for determination of intracellular bacteria number. Samples taken from water lysates were serial diluted and spread on Beef blood agar. Colonies were counted following one day of incubation at 37˚C. RA1 lysates were stored at -70 ˚C freezer prior to isolation of RNA and gene expression analyze.

3.1.4. Induction of inflammation by LPS on HC11 cells

To induce experimental inflammation on HC11 cells, Two T75 Flasks of differentiated cells were used. Cells in one flask were treated with differentiating medium containing LPS 10 µg/mL (Sigma-Aldrich, St. Louis, USA) for 48 hours. Control cells were treated with differentiating medium without LPS. These two flasks were incubated at 37 ˚C and 5% CO_2.

3.1.5. Cell toxicity test for LPS-treated cells

Medium Samples from both LPS and control cells were taken and stored at -20 ˚C freezer. NADH stock solution (175 µl at concentration of 6.6 mM) was added to 4.65 ml of Tris HCl buffer (at concentration of 0.2mM and pH 7.3) and mixed thoroughly. 195 µl of this solution was added to 10 µl of medium sample (each samples were in duplicate). The mixture (loaded in 96 well-plate) were incubated at room temperature for 15 minutes and then at 25 ˚C in a microplate reader Victor^2 1420 multilabel counter (Perkin Elmer, Wellesley, USA). 15 µl of sodium pyruvate (30mM) was added to each well and absorbance values at 340nm recorded 6 times/well. Lactate dehydrogenase

activity in the collected medium samples was calculated by the formula presented below:

LDH(U/mL)= ΔA per minute/ (0.001 x 0.1x 0.8)

3.2.RNA isolation

Total RNA was extracted from cells or tissue samples by the use of Nucleospin® RNA II kit (Macherey-Nagel, Düren, Germany). Mammary tissues were obtained from previous experiments with lactating mice approved by the Local Ethics Committee of Animal Research (permit no. C159/2).The whole procedure was performed according to manufacturer's manual; every 30 mg of tissue sample or $5x10^6$ of cultured cells was lysed by RA1 buffer (containing 1% β-mercaptoethanol) and RNA precipitated by addition of 70% ethanol. The lysate added to a filtered column and washed by membrane desalting buffer. DNA was digested by addition of DNase I reaction mixture (containing 10% reconstituted rDNase) and incubated for 15 minutes at room temperature. The bound RNA washed by RA2 and RA3 buffer and finally eluted at 60 or 50 µl of RNase-free H_2O.

3.3.Total RNA quantification and RNA gel electrophoresis

Quantification of total RNA isolated from cells or tissue was carried out by the use of Ribo-Green ® RNA Quantification Kit (Molecular Probes, Invitrogen, Eugene, USA). RNA concentration was determined by following the instruction of manufacturer's manual. Ribosomal RNA was used as a RNA standard at concentrations of 0.02, 0.1, 0.5 and 1 µg/ mL loaded in duplicates and diluted samples were loaded in triplicates in 96 well plates (dilution rate was 200 to1600 times, depending on the concentarion of the isolated RNA). Fluorescence was measured in Victor^2 1420 multilabel counter (Perkin Elmer, Wellesley, USA). The equation obtained by the RNA standards was used to calculate concentration of samples.

The integrity of RNA after isolation was checked by running at least 300 ng of RNA sample on 1% agarose (Sigma-Aldrich, St. Louis, USA) at 60 V for 90 minutes. Two bands of ribosomal RNA were observed as in indication of intact RNA (28s and 18s rRNA) (Figure1).

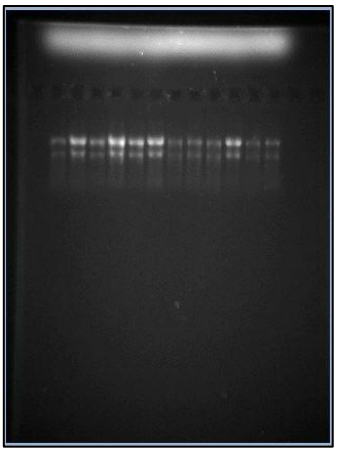

Figure 1. RNA gel electrophoresis of isolated RNA from HC11 cells differentiated according to first cell differentiation protocol; two distinct rRNA bands in gel are an indication of intact RNA. Lane 1-6 RNA from undifferentiated cells & 6-12 differentiated cells.

Concentration of RNA standard which were used to draw the standard curve is shown in table 1 at bellow.

Table 1-RNA standard concentration and their fluorescence after measurement in Victor, values from duplicates were used to calculate average and then background was subtracted from them obtaining true fluoresce.

Standard RNA concentration (ng/mL)	Duplicate 1	Duplicate 2	average	True fluorescence
0	2473	2126	2299,5	0
20	8531	8865	8698	6398,5
100	38630	40312	39471	37171,5
500	179692	183349	181520,5	179221
1000	316344	329991	323167,5	320868

Values from table 1 were used to draw the following graph. One equation was obtained from standard curve (Figure 2) which was used for calculation of RNA concentrations in unknown samples.

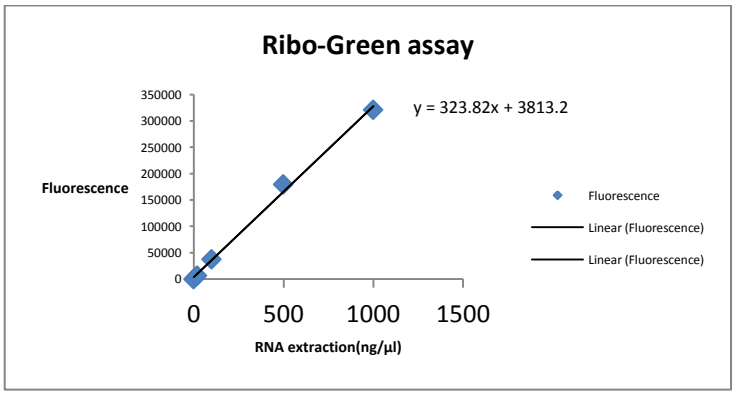

Figure 2. Standard curve of Ribo- Green assay; equation of regression line was used for fluorescence values of known samples to calculate RNA concentrations.

Concentration of RNA in unknown samples were calculated according to the equation shown in figure 2 , an example of calculated concentrations for the RNA isolated from

HC11 cells treated with cell differentiation I passage number 31 is shown below in table 2.

Table 2. RNA samples measured by Ribo-Green assay, each sample was loaded as triplicate, mean values were calculated and corrected for background and concentration obtained according to equation.

Samples name	Mean Fluorescence value of samples	Correction for background	RNA concentration(ng/mL)
1	29868.3	27568.8	117.376
2	123517	121217.5	580.096
3	85164	82864.5	390.592
4	79701	77401.5	363.600
5	42378.3	40078.8	179.072
6	54117	51817.5	237.184
7	28043.6	25744.1	108.3609
8	22933	20633.5	83.104
9	23894.3	21594.8	87.856
10	21119.6	18820.1	74.144
11	33794	31494.5	136.768
12	26997.6	24698.1	103.184

3.4.Primer Design and DNA gel electrophoresis

Primers were designed to detect expression of two genes in samples; Slc22a4 (solute carrier family 22, member4 (Gene ID: 30805)) encoding Organic cation transporter protein, novel, type1 (OCTN1) and Slco1a5 (solute carrier organic anion transporter family, member 1a5 (Gene ID: 108096)) Organic anion transporting polypeptide 3(OATP3)**.** To design primers cDNA of these genes were obtained from NCBI database (www.ncbi.nih.gov) and intron-spanning primers picked in Primer 3 software to have PCR product of 150-200 base pairs. Primers were generated by Cybergene Company (Stockholm, Sweden). Sequences of primers are listed below in table 3.

Table 3- OCTN1 and OATP3 primer sequence

Protein	Primer Sequnce
OCTN1	5’-CCTGTTCTGTGTTCCCCTGT-3’ (Forward) 5’- GGTTATGGTGGCAATGTTCC-3’ (Reverse)
OATP3	5’-GCACAGAGAAAAAGCCAAGG-3’ (Forward) 5’- CTCCAGGTATTTGGGCAAGA-3’ (Reverse)

PCR product of these primers were run on 1% agarose at 60 V for 90 minutes, to check if primers attach specifically and their products have the right size.

3.5.Reverse Transcription real-time PCR:

To study gene expression in HC11 cell line or tissue samples, QuantiTect ®SYBR ® Green RT-PCR kit (Qiagen, Hilden, Germany) was used. Each sample was run in duplicate containing 100 ng of template RNA sample and 0.4 µM of primer in 25 µ l of reaction mix. PCR program was; 50 ˚C – 30 minutes, 95˚C – 15 minutes, 40 cycles (each cycle 94˚C- 1 minute, 55˚C- 1 miunte, 68˚C- 45 seconds), 68˚C- 7 minutes. Melt curve analysis from 50˚C to 99˚C was performed in direction to the RT-PCR. Both RT-PCR and melt curve analyses were performed by using a Rotor Gene (RG3000, Corbett Life Science, Sydney, Australia).

One example of melt curve generated by Rotor gene is shown below (Figure3). Melt curves were used to check accuracy of PCR runs in by observing the melt peak at the same temperature.

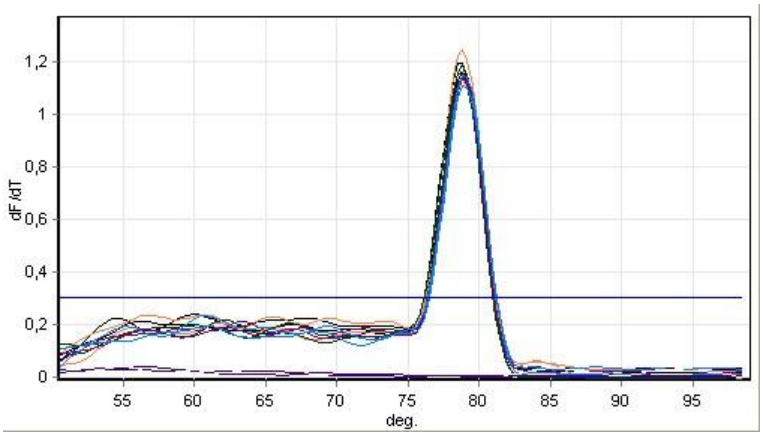

Figure 3: Melt curve of β-casein expression in HC11 cells differentiated according to cell differentiation protocol II .100ng of RNA template was used (passage number 33), differentiated and undifferentiated cells RNA were loaded in duplicates (N=3).

3.6. Statistical analysis:

To determine if there has been any statistically significant difference between treated and un- treated samples all data obtained from qRT-PCR has been analyzed by MiniTab 16 Statistical software, and reported as Mean ± standard deviation. Two set of data analyzed by ANOVA test (one-way unstacked); difference between sets of data is significant when p value is ≤ 0.05.

4. Results

4.1. Gene expression of transporter in mouse mammary tissue

As a quality control, the PCR gene expression of transporters was investigated in mouse mammary tissue isolated in different days during lactation; day 2 (early lactation), day 7(peak lactation) and day 20 (late lactation -involution). For this experiment mouse mammary tissue isolated from one individual was used in duplicates to obtain expression pattern of transporters during lactation. Day 2 was chosen as a standard for basal expression and all expression values were normalized to day 2. No statistical analysis was performed for these data.

β- casein gene expression was upregulated through early lactation to peak lactation, but downregulated at the end of lactation (Figure 3). *Bcrp* gene expression was upregulated from early to peak lactation but it was slightly decreased through end of lactation (Figure 4), while *Mdr1* gene expression was dramatically downregulated from early to peak lactation but it was slightly increased through end of lactation (Figure 5). *Mrp1* gene expression remained at the same level as lactation day 2 through lactation with a weak tendency for upregulation at day 7 (Figure 6). *Oatp3* gene expression was dramatically downregulated from early to peak lactation but it began to increase through end of lactation (Figure7). *Octn1* gene expression did not change from early to peak lactation but it decreased through end of lactation (Figure 8).

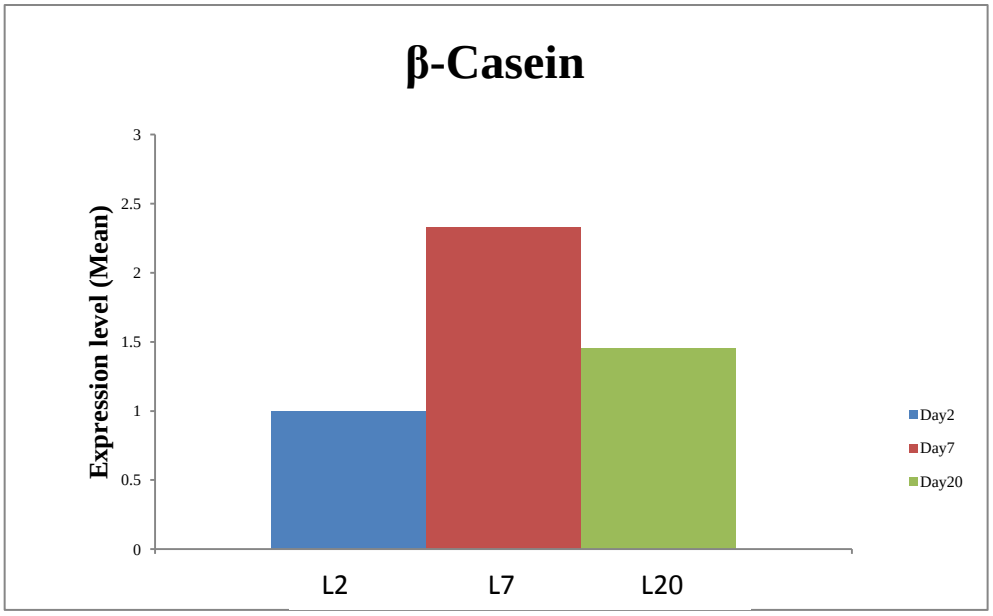

Figure 3. β-casein expression in mammary tissue of lactating mouse. Mean value of each lactation day was normalized to day 2.

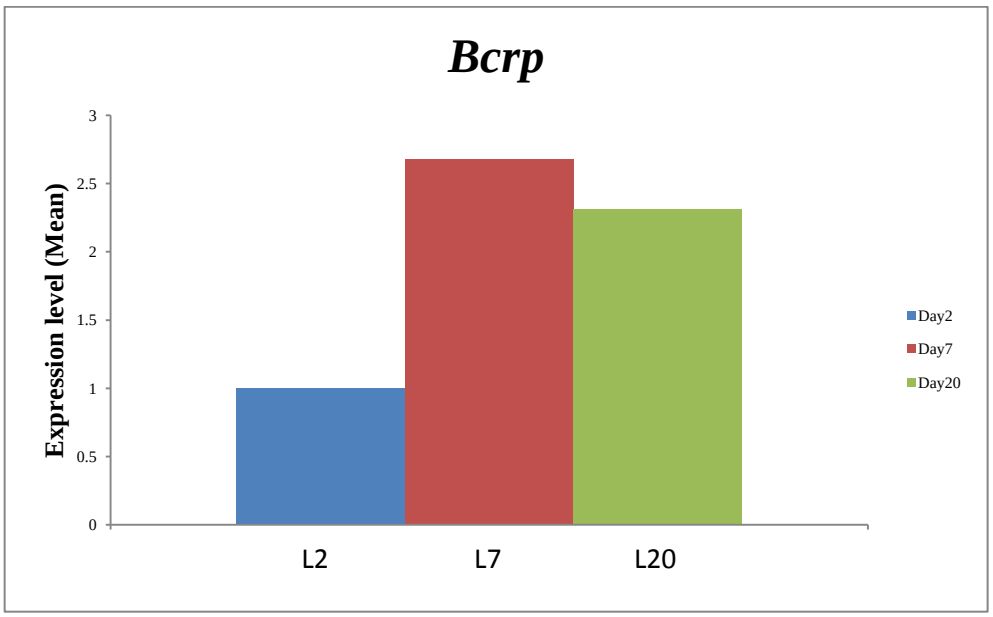

Figure 4. *Bcrp* expression in mammary tissue of lactating mouse. Mean value of each lactation day was normalized to day 2.

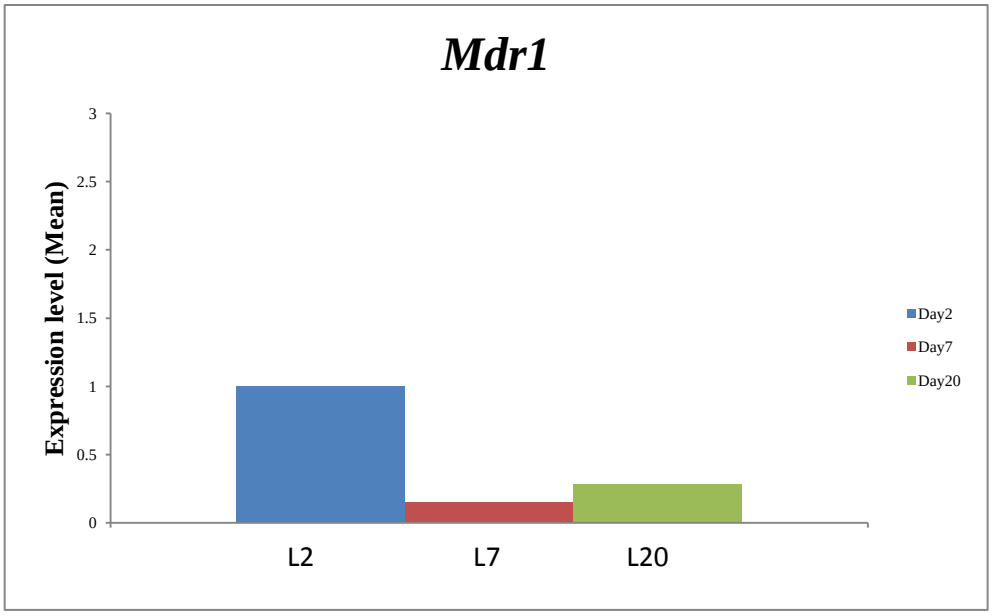

Figure 5. ***Mdr1*** **expression in mammary tissue of lactating mouse. Mean value of each lactation day was normalized to day 2.**

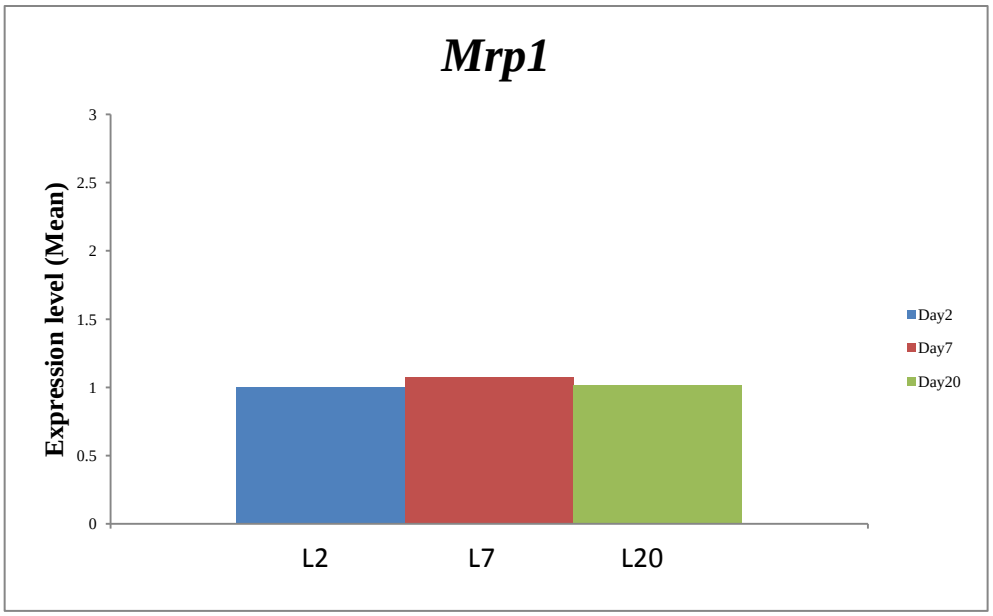

Figure 6. ***Mrp1*** **expression in mammary tissue of lactating mouse. Mean value of each lactation day was normalized to day 2.**

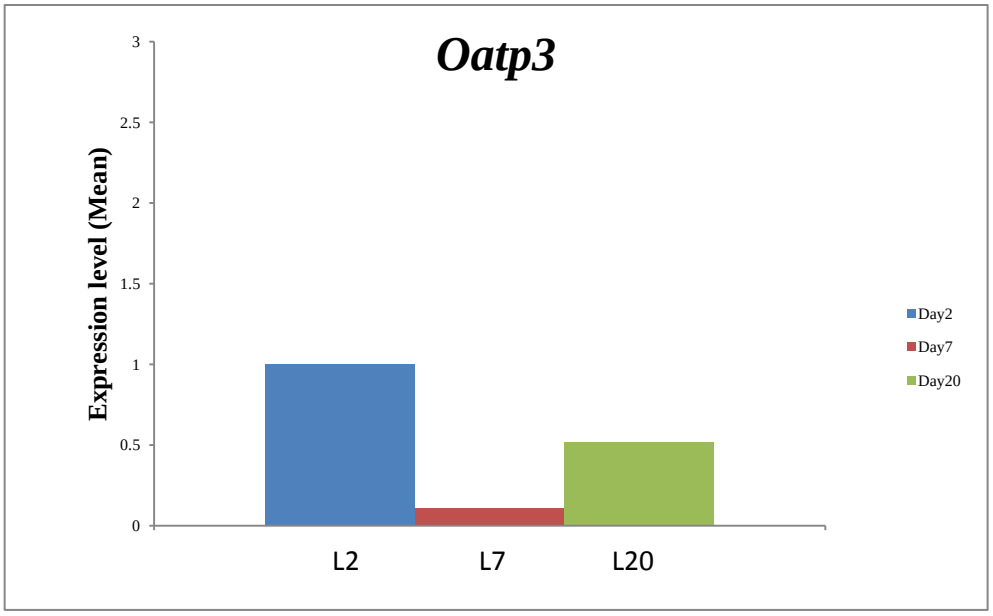

Figure7. *Oatp3* expression in mammary tissue of lactating mouse. Mean value of each lactation day was normalized to day 2.

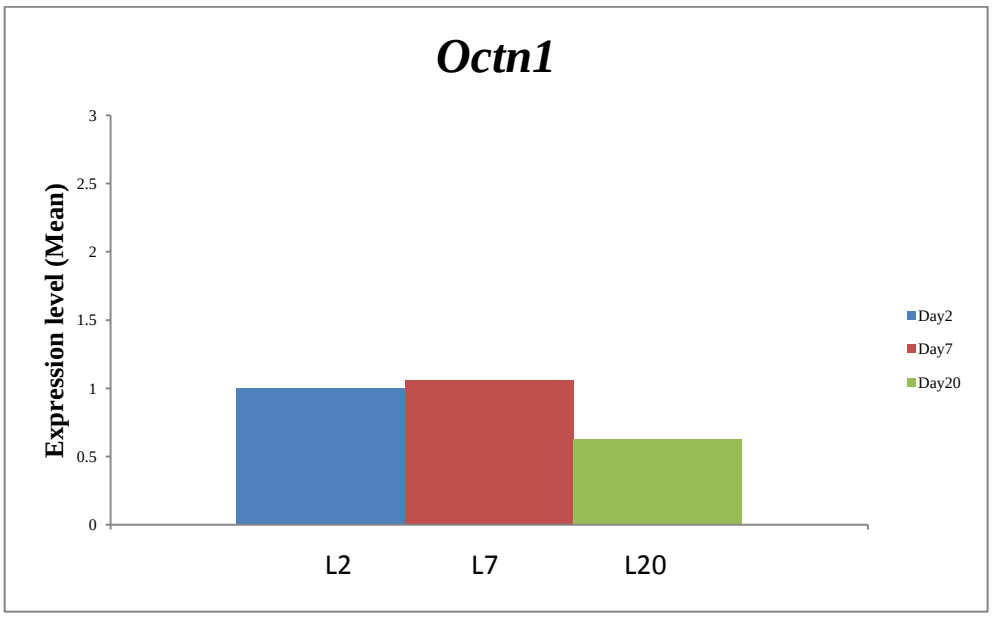

Figure 8. *Octn1* expression in mammary tissue of lactating mouse. Mean value of each lactation day was normalized to day 2.

4.2. Gene expression of transporters in cell differentiation protocol I and II

HC11 cells passage number 31 and 33 were differentiated to obtain secreting phenotype by protocol I and II, respectively and protocols were compared in order to find out which protocol induces differentiation better. These two protocols are different in respect of differentiation time. HC11 cells were differentiated during 7 days post-confluency in protocol II, compared to because 3 days in protocol I.

β- casein expression was analyzed in cells as a marker of differentiation of HC11 cells into a secreting phenotype. Both protocols induce β- casein upregulation but protocol I provoked upregulation about 30 folds higher than protocol II (Figure 9). *Bcrp* gene expression was studied in HC11 cells differentiated by both protocols I and II. In HC11 cells differentiated by either of protocols, *Bcrp* expression was downregulated (Figure 10). *Mdr1* expression was significantly downregulated in HC11 cells when they were differentiated by protocol II, but no difference in expression was observed for HC11 cells differentiated according to protocol I (Figure 11). *Mrp1* expression was significantly downregulated in HC11 cells when they were differentiated by protocol I, but its expression did not change after differentiation by protocol II (Figure 12). *Oatp3* expression was significantly upregulated in HC11 cells when they were differentiated by protocol I (about two fold increase in expression), but its expression in HC11 cell differentiated according to protocol II showed a tendency to be downregulated although no statistically significant difference in expression was obtained from F test analysis (ANOVA, one-way unstacked) (Figure 13). *Octn1* expression was significantly downregulated in HC11 cells differentiated by either of protocols which is contrary to in vivo studies, but downregulation in cells differentiated according to protocol I was more (less than half of the expression of *Octn1* in control cells) than protocol II (Figure 14).

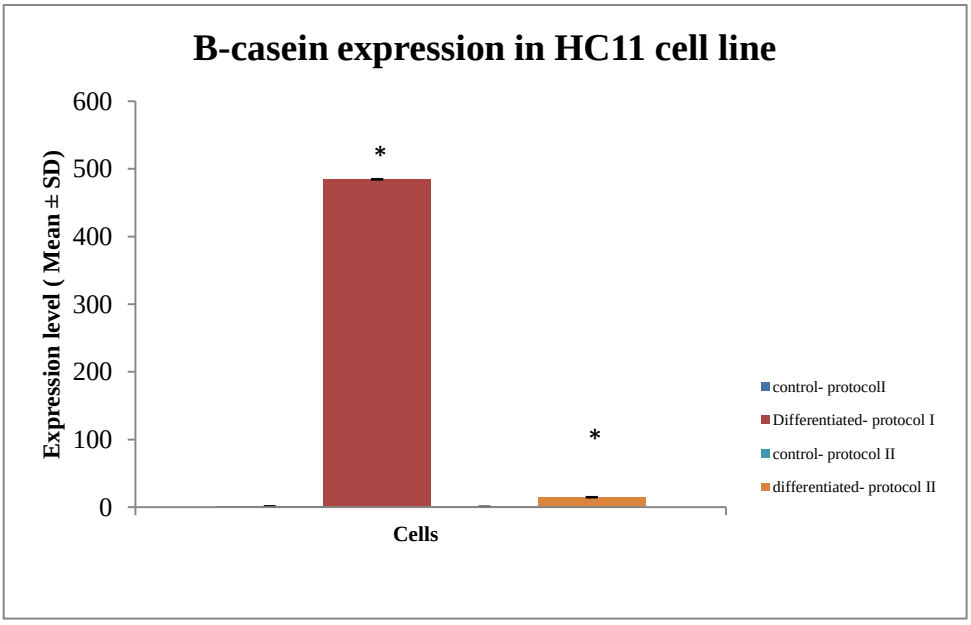

Figure 9. Comparison of β-casein expression in HC11 cells. Mean values and standard deviations of control and differentiated cells for both protocol I and II are shown on graph after normalization (N=6, N=3 for protocol I and II respectively). (* P value ≤0,05)

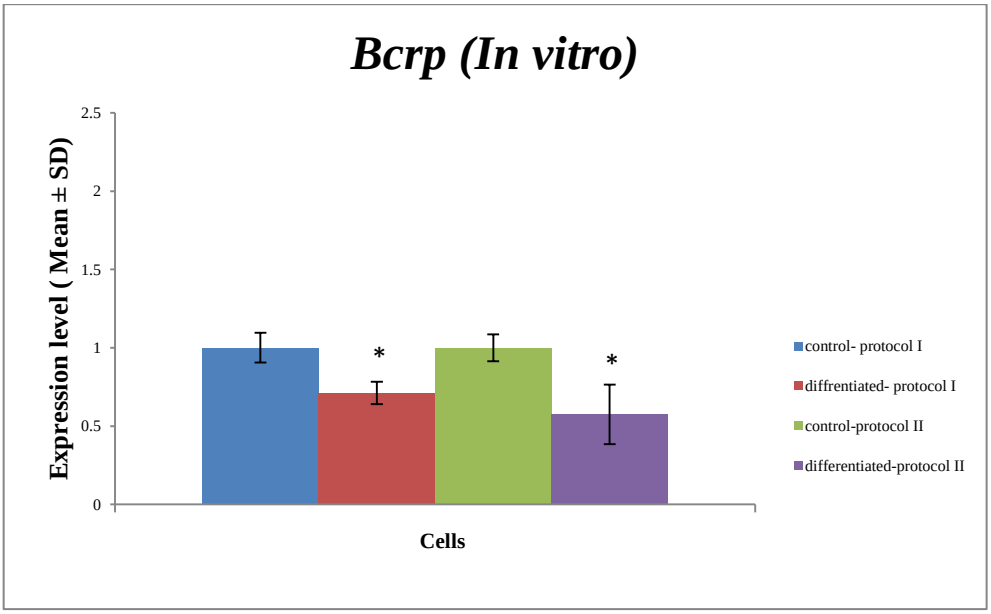

Figure 10. Comparison of *Bcrp* expression in HC11 cells. Mean values and standard deviations of control and differentiated cells for both protocol I and II are shown on graph after normalization (N=6, N=3 for protocol I and II respectively). (* P value ≤0,05)

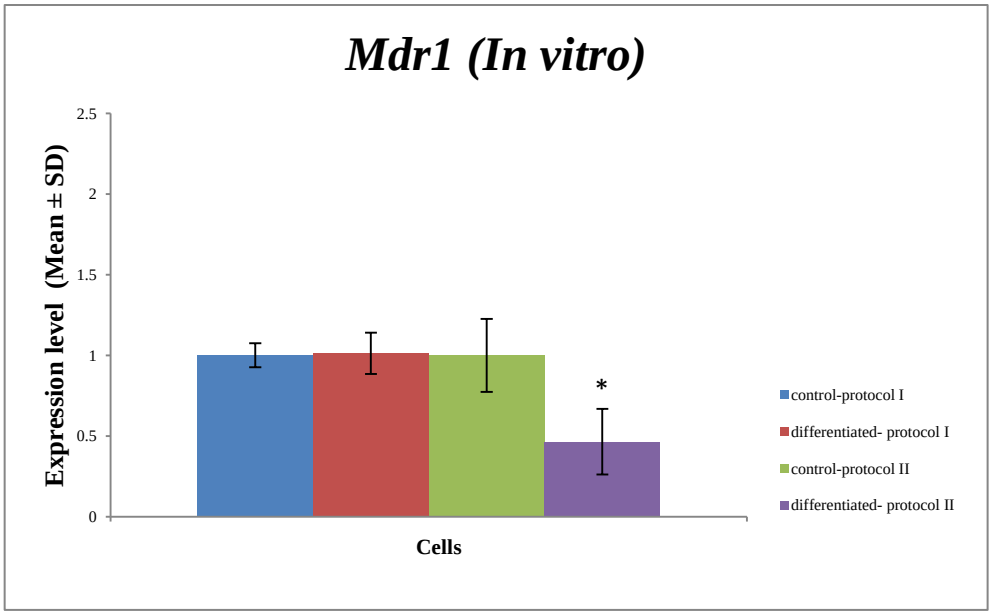

Figure11. Comparison of *Mdr1* expression in HC11 cells. . Mean values and standard deviations of control and differentiated cells for both protocol I and II are shown on graph after normalization (N=3, N=3 for protocol I and II respectively). (* P value ≤0, 05)

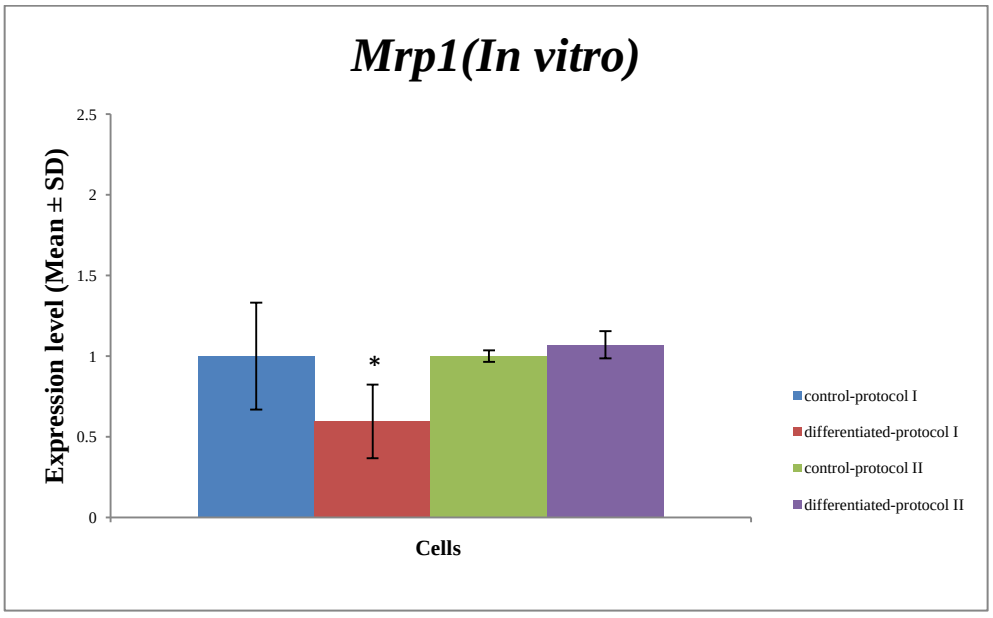

Figure 12. Comparison of *Mrp* 1 expression in HC11. Mean values and standard deviations of control and differentiated cells for both protocol I and II are shown on graph after normalization (N=6, N=3 for protocol I and II respectively). (* P value ≤0, 05)

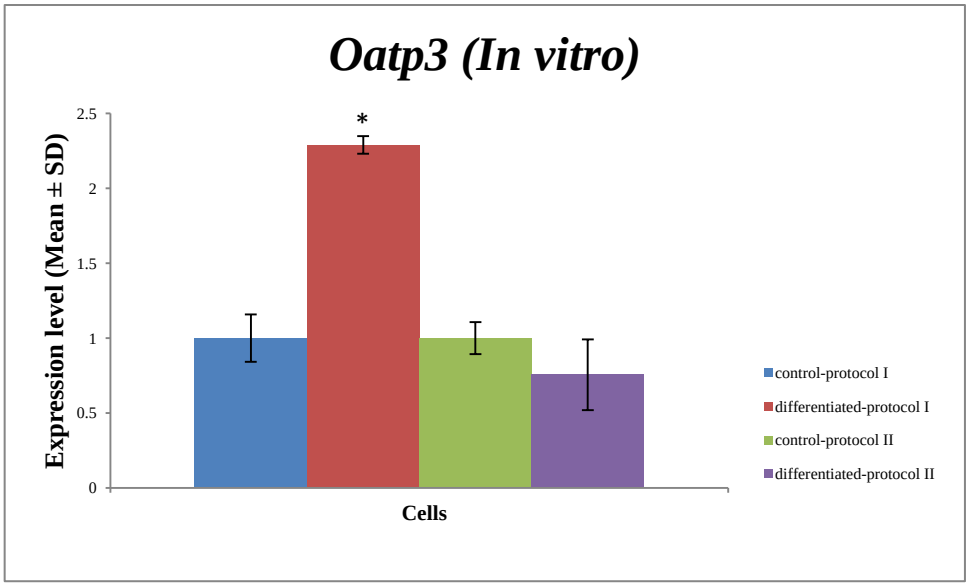

Figure 13. Comparison of *Oatp3* expression in HC11 cells. Mean values and standard deviations of control and differentiated cells for both protocol I and II are shown on graph after normalization (N=3, N=3 for protocol I and II respectively). (* P value ≤0, 05)

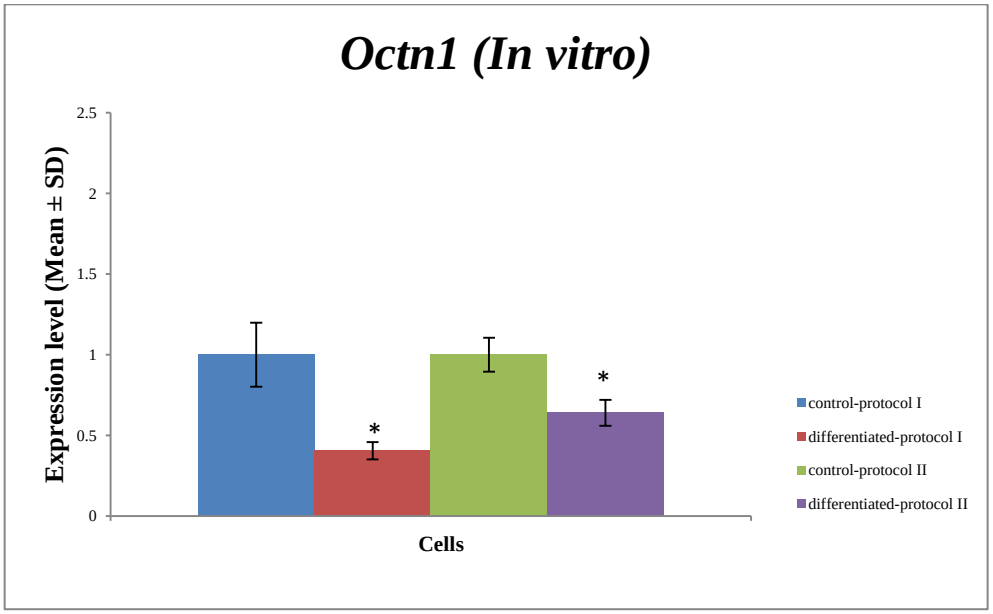

Figure 14. Comparison of *Octn1* expression in HC11 cells. Mean values and standard deviations of control and differentiated cells for both protocol I and II are shown on graph after normalization (N=3, N=3 for protocol I and II respectively). (* P value ≤0, 05)

4.3. Gene expression of transporters during subclinical mastitis and LPS treatment

To investigate the impact of subclinical mastitis on transporters gene expressions, HC11 cells (passage number 31) were infected by *Staphylococcus aureus* and RNA was isolated. HC11 cells (passage number 36) were treated by LPS to simulate *E.coli* mastitis for investigation of any potential impacts on transporters gene expression.
To check if LPS treatment had any lethal effect on cells by damaging cell membrane structure, cell toxicity test was performed.
LDH concentration was measured and the obtained results are displayed in Figure15.
LDH concentration was not increased in HC11 cells treated by LPS.

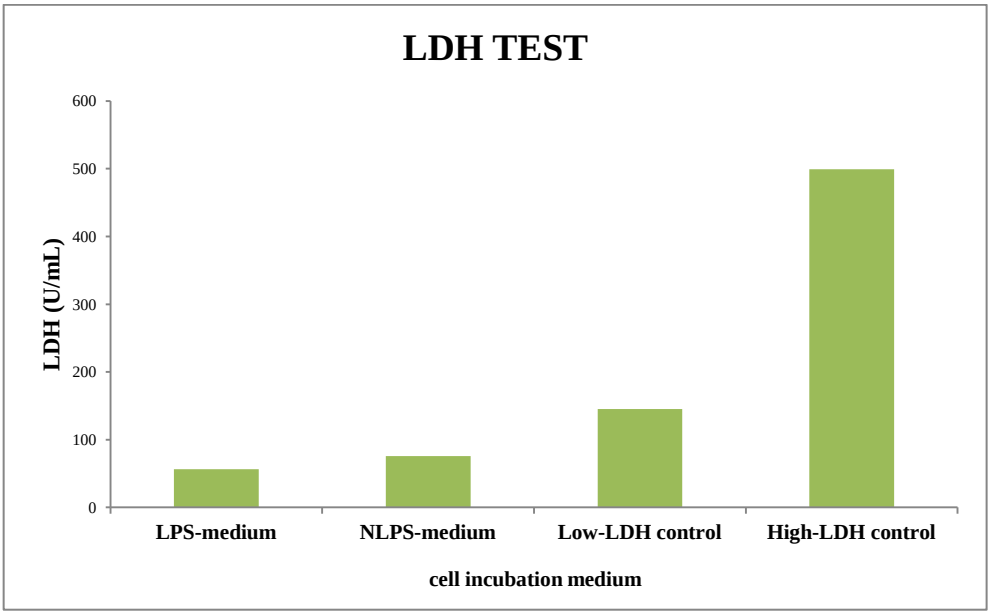

Figure 15. Results of cell toxicity test on HC11 cells, passage number36. LDH concentration was measured as an indicator of cell membrane damage in medium from LPS-treated cells and from controls = Non-LPS-treated (NLPS). Both media had lower LDH concentration than low- LDH control.

Cell toxicity test had been performed on HC11 cells infected by *S. aureus* previously in the lab, and no cell membrane damage was detected in HC11 cells on that assay but the results are not included.
β-casein gene expression was studied in differentiated HC11 cells in both *S.aureus* infection and LPS treatment. At infection state, it showed a tendency for upregulation without any statistically significant difference, while LPS treatment seemed to have no especial effects on its expression (Figure16). *Bcrp* and *Mdr1* expressions in both

infected HC11 cell and LPS –treated did not change recognizably (Figure 17 & 18). *Mrp1* expression also did not change by either *S.aureus* or LPS treatment (Figure 19). *Oatp3* was upregulated significantly after LPS treatment comparing to control cells but no change was detected in *S.aureus* infected cells (Figure20). *Octn1* was upregulated in presence of *S. aurues* but no clear tendency was observed in LPS treatment (Figure21).

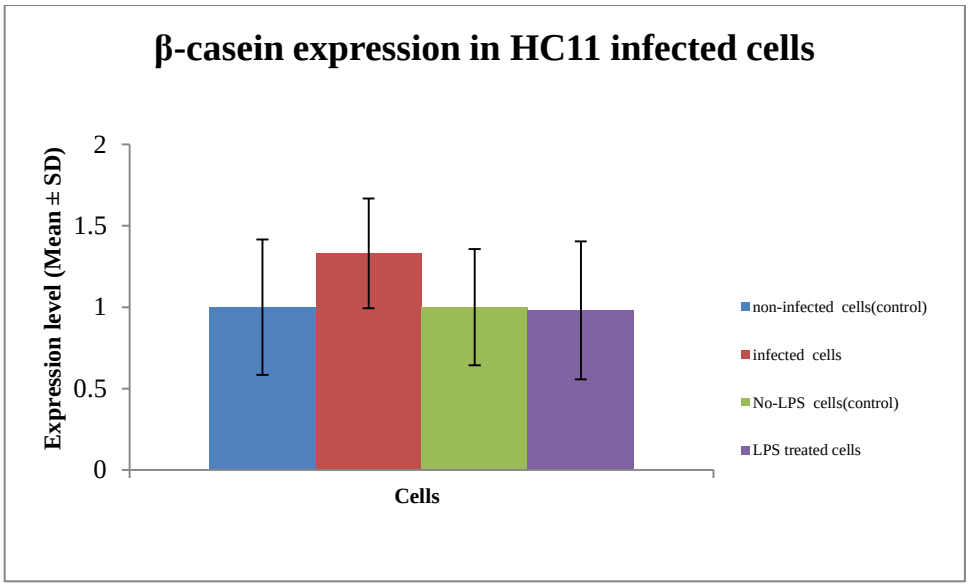

Figure 16. β-casein expression in HC11 cells in presence of *S. aureus* & LPS. Mean values and standard deviations of control and treated cells are shown on graph after normalization (N=3, two controls were included in *S.aureus* HC11 cells).

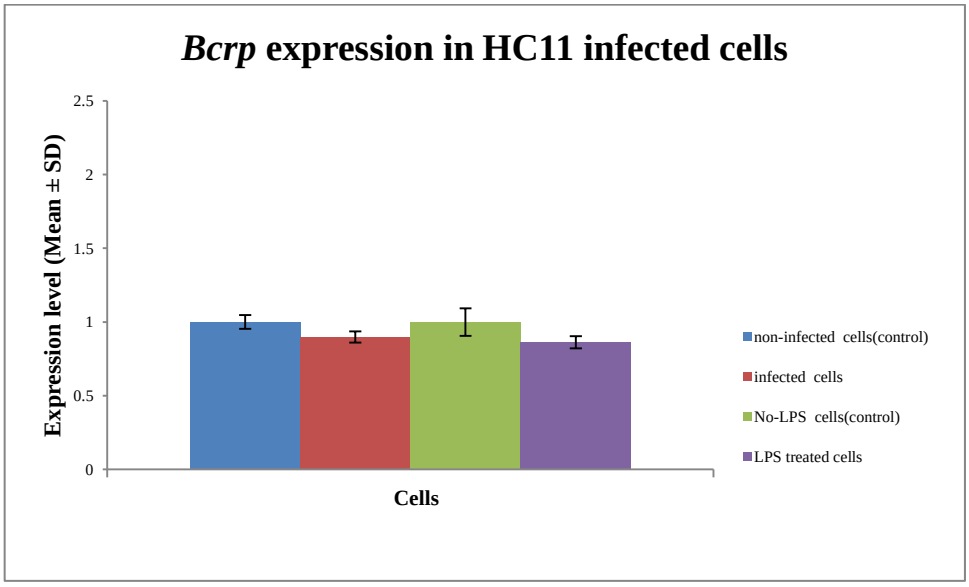

Figure 17. *Bcrp* expression in HC11 cells in presence of *S. aureus* & LPS. Mean values and standard deviations of control and treated cells are shown on graph after normalization (N=3, two controls were included in *S.aureus* HC11 cells).

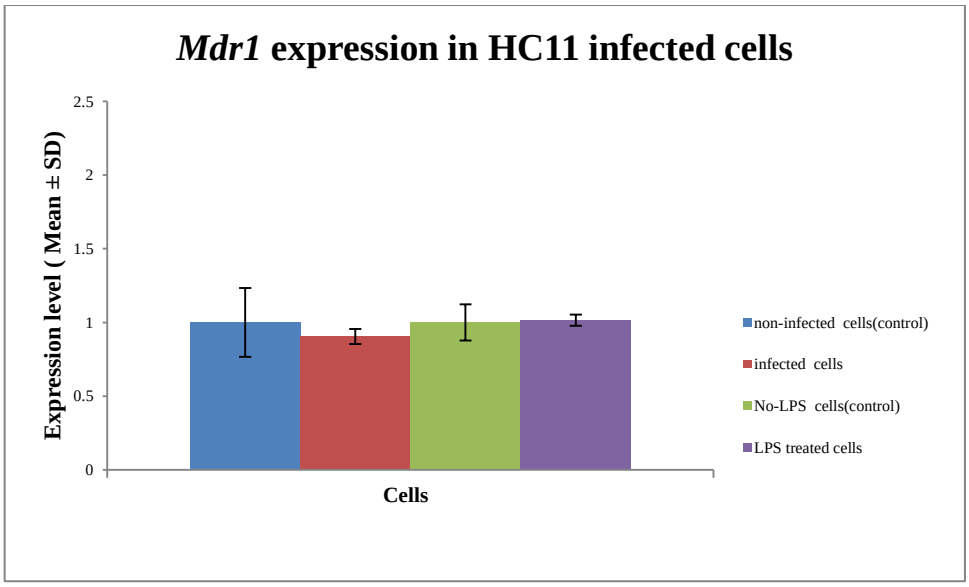

Figure 18. *Mdr1* expression in HC11 cells in presence of *S. aureus vs* LPS. Mean values and standard deviations of control and treated cells are shown on graph after normalization (N=3, two controls were included in *S.aureus* HC11 cells).

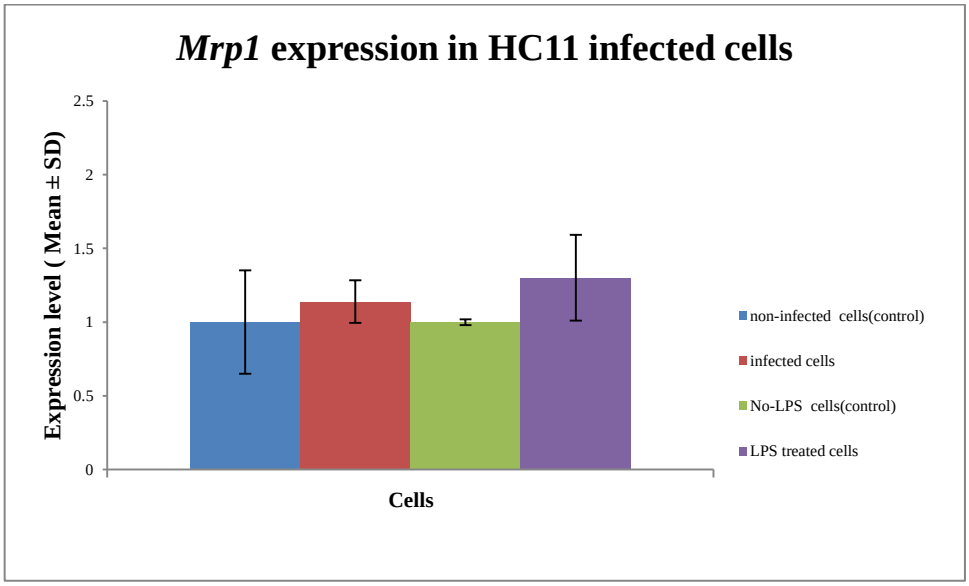

Figure 19. *Mrp1* expression in HC11 cells in presence of *S. aureus* vs LPS. Mean values and standard deviations of control and treated cells are shown on graph after normalization (N=3, two controls were included in *S.aureus* HC11 cells).

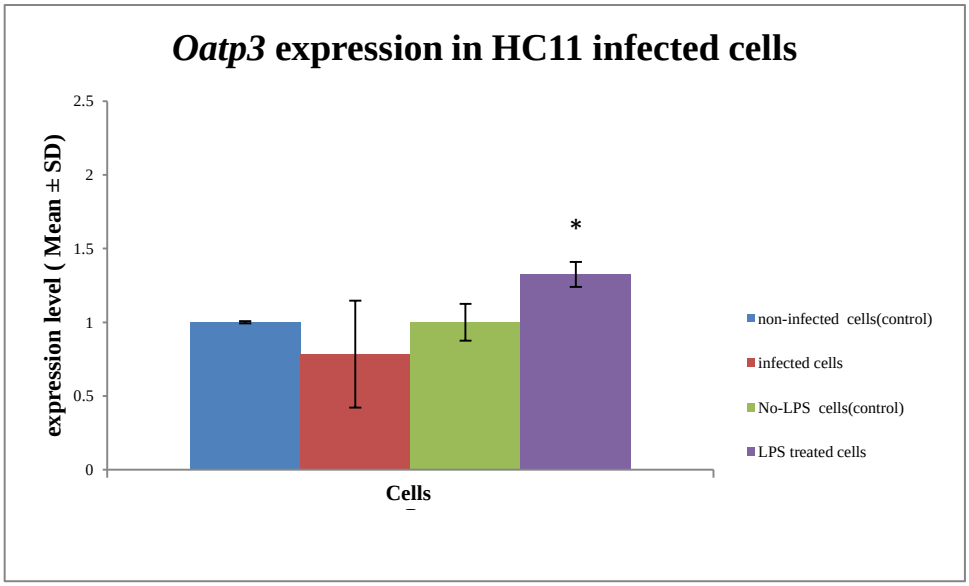

Figure 20. *Oatp3* expression in HC11 cells in presence of *S. aureus* & LPS Mean values and standard deviations of control and treated cells are shown on graph after normalization (N=3, two controls were included in *S.aureus* HC11 cells) . It was upregulatedin presence of LPS but it showed a tendency for downregulation in presence of *S. aureus* (* P value ≤0, 05).

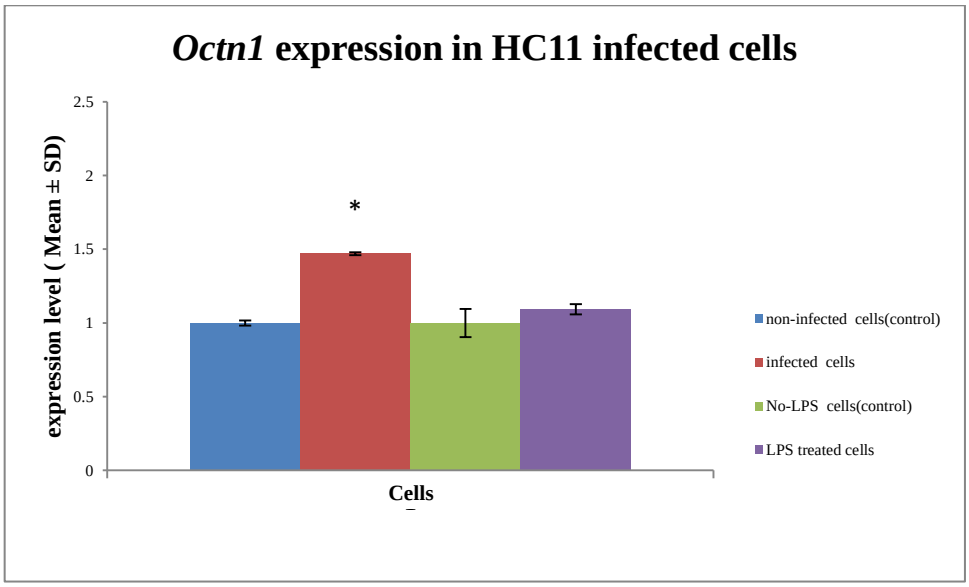

Figure 21. *Octn1* expression in HC11 cells in presence of *S. aureus* & LPS Mean values and standard deviations of control and treated cells are shown on graph after normalization (N=3, two controls were included in *S.aureus* HC11 cells). It was evidently upregulated in presence of *S. aureus* (* P value ≤0, 05).

5. Discussion

In this study, gene expression of several membrane transporters in the murine mammary epithelial HC11 line was investigated, when HC11 cells were differentiated by two

different protocols. Also gene expressions of them were studied when differentiated cells were infected by *S. aureus* or exposed to LPS to simulate subclinical mastitis.

As a quality control of the PCR technique, we studied gene expression of target transporters in murine mammary tissues isolated from lactating mouse and expression levels of transporters were studied in tissues from lactation day 2, 7 and 20. Some genes showed upregulation through lactation, while some were downregulated or their expression did not change during lactation. β-casein, and *Bcrp* were highly upregulated, while *Mdr1* and *Oatp3* were strongly downregulated during lactation, but *Octn1* was downregulated at the end of lactation. *Mrp1* did not display any change in expression level.

All data of *in vivo* experiment, was in complete accordance with previous studies performed by other research groups. *Bcrp1* gene expression was strongly upregulated in mouse mammary gland. Immunohistochemical and western blot studies of *Bcrp1* by Jonker et al. showed the same result (18). *Mdr1* gene expression was found downregulated in our study, which is in line with reports of its downregulation in mammary gland of humans and rats through lactation (26, 27). *Mrp1* gene expression did not show any change from lactation day2 till day 20, while in the study by Alcorn et al. (26) on lactating rat mammary gland its expression was downregulated at the beginning of lactation and remained at low levels through it. Since we did not include pregnant mouse mammary tissue at late gestation, no comparison between its expression levels in lactation with pregnancy period could be performed to confirm a true downregulation.

Oatp3 gene expression, to my knowledge, was studied for the first time in murine lactating mammary gland and its expression was shown to be decreased through lactation while *OATP-A* (its human ortholog) was reported to be upregulated in epithelial cells of human mammary tissue during lactation (27). *Octn1* expression in a study by Alcorn et al. was reported to be upregulated from parturition till day 3 in rat mammary gland but it decreased through end of lactation (26), while in our study the *Octn1* expression was highest in day 7. The difference between results of this study and Alcorn's can be result of intense upregulation of *Octn1* between day 2 and day 3 of lactation which was skipped in this study.

5.1. Cell differentiation protocol effect on gene expression of transporters

In this study, we used two different cell differentiation protocols to induce secreting phenotype in HC11 cells, to optimize an in vitro model for membrane transporters during lactation. In both protocols prolactin, glucocorticoids and insulin were added to cells to induce terminal differentiation but protocols was different in respect of timing. In protocol I cells were differentiated 4 days post-confluency, while in protocol II cells were differentiated after 7 days post-confluency.

We investigated gene expression of transporters in differentiated HC11 cells to compare their potential similarity to *in vivo* conditions. β-casein upregulation is a marker for secreting phenotype in mammary cell lines. Differentiation of cells according to protocol I resulted in a stronger expression of β-casein than protocol II (30 folds higher expression was detected in HC11 cells differentiated by protocol I). Detection of such considerable expression difference between the two protocols can be result of allowing cells to be confluent for 3 days more in protocol II than protocol I. It should be mentioned that, HC11 cells were primarily isolated from mouse at mid-pregnancy, when β-casein expression is already elevated. Since, HC11 cells according to protocol II were allowed to stay in growth medium for longer time prior to differentiation comparing to protocol I, β-casein expression might increase further in control cells leading to detect higher expression level of β-casein in protocol II control cells. Consequently, the relative expression fold difference between control and differentiated cells reduces which leads to detection of less gene expression increase in return.

Some transporters expression was in accordance with previous studies, while others do not show the expected expression pattern.

*Bcrp1, Mdr1*and *Octn1* did not show the expected expression pattern in differentiated HC11 cells. *Bcrp1* expression decreased in HC11 cells, which was in contrast to *in vivo* studies that reported its upregulation (18). *Mdr1* was supposed to be downregulated in mammary epithelial cells while its expression did not change after differentiation (26). Alcorn et al. reported its notable downregulation in mammary epithelial cells isolated from human tissue (27). *Mrp1* expression was downregulated in our study, as previously reported in studies on rat mammary tissue and human mammary epithelial cells (26, 27).

Octn1 was expected to be upregulated in HC11 cell based on previous findings (26, 27, 34), while it was obviously downregulated in our experiments. Alcron et al. reported its

upregulation in human mammary epithelial cells (27). *Oatp3* has not been studied in mouse, yet its human ortholog (*OATP-A*) was reported to be upregulated in Alcorn et al. study in human mammary epithelial cells during lactation (27). In our study, its expression in murine tissue was downregulated at peak lactation (day 7). Since, differentiated HC11 cells are supposed to display peak lactation phenotype, it might be concluded that downregulation of *Oatp3* in HC11 cells was in complete accordance to *in vivo* situation when it was found downregulated at day 7.

Membrane transporters genes in differentiated HC11 cells did not follow the *in vivo* studies pattern for *Bcrp1*expression, which was reported to be markedly upregulated, in fact, its expression in differentiated HC11 cells by both protocols was downregulated in our study. *Mrp1* expression did not change in differentiated HC11 cells by protocol II. Its expression in both rat mammary tissue and human mammary epithelial cells was downregulated during lactation (26, 27).

Mdr1 was downregulated like *in vivo* studies on rat mammary gland (26). *Octn1* displayed similar expression patterns in HC11 cells differentiated according to either protocol I or II. *Oatp3* showed a tendency for downregulation without any statistically significant difference with control cells due to large standard deviation.

As a result of obtaining unexpected expression level of transporters in differentiated HC11 cells by either protocol I or II, it is difficult to choose either of protocols as an optimal method for differentiation. Several reasons can be suggested for detection of different gene expression in differentiated HC11 cells. First of all, gene expressions of transporters have been usually studied *in vivo*, which includes RNA sample of a conglomerate of different cell types within tissue. Each cell type might have distinctive expression for membrane transporters, while RNA contents of all cell types mix during RNA isolation procedure and target mRNA may dilute or concentrate in the final aliquot. Furthermore, post-transcriptional regulation has been proposed to be a very prominent process in HC11 cells especially during differentiation that may result in difference between mRNA and protein level at time of detection (41).

5.2. *S.aureus* infection and endotoxins effect on gene expression of transporters

Very few studies have been conducted on transporter gene expression during mastitis and information on gene expression of transporters is not available extensively. We

tried to simulate infection condition in an *in vitro* cell model (HC11 cells) and investigate potential impact of *S. auerus* or *E.coli* infection on membrane transporters expression. We used differentiated HC11 cells by protocol II and treated them with *S. aureus* and LPS.

Most of transporters showed no significant difference in expression in either of conditions. This can be due either to a too low dose of LPS or that genes are not affected by LPS. In infection experiment by *S.aureus,* the only affected transporter was *Octn1* which was upregulated. β-casein expression displayed a tendency for upregulation in presence of *S.aureus*.

In LPS treated HC11 cells just *Oatp3* showed alteration in expression pattern and it was upregulated in presence of endotoxins. Also *Mrp1* displayed a tendency for upregulation but standard deviation of data was too large to be considered statistically significant. *Mrp1* mRNA and protein concentrations were studied by Vos et al. (42) and it showed upregulation at both levels after 6 hours exposure to LPS. β-casein secretion in mastitic dairy cows (*E.coli* infection) was shown to be increased by Boehmer et al. (43). But this had been reported by measuring the protein and cannot be accepted as a clear indication of gene upregulation, since possible post-translational regulation mechanisms may interfere. We could not detect its upregulation in LPS experiment. *Mdr1* expression had been checked in epithelial cells of small intestine and liver cells during mastitis. In both tissues its expression was downregulated and its efflux activity impaired (44, 45) but we did not detect any change of its expression. Octn1 as an $_{L}$-carnitine transporter was studied in rat mammary gland by Alcorn et al., during induced LPS mastitis (46) and it was observed that *Octn1* was upregulated at lactation day 4 and day 11.

6. Conclusion

HC11 cells were demonstrated to express all the investigated membrane transporters. Some differences between the two evaluated differentiation protocols were detected, regarding gene expression of the membrane transporters. However, a common observation for both differentiation protocols was a downregulation of *Bcrp* and *Octn1*. Bacterial infection of HC11 cells resulted in upregulation of *Octn1,* whereas LPS treatment induced upregulation of *Oatp3*. Further studies are needed to elucidate the physiological role of reduced *Bcrp* and *Octn1* in secreting HC11 cells and also to confirm the upregulation of *Oatp3* in LPS treated cells and *Octn1* in *S. aureus* infected cells.

7. Acknowledgements

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