Transport Proteins in Mammary Epithelial Cells

Studies in Murine (HC11) and Bovine (BME-UV) Cells – Effects of Prochloraz

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Cover: Histological sections of mouse mammary glands at various stages and a photo of a Simmental cow with a suckling calf (Y. Yagdiran and E. Ozsenturklu).

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Abstract

Transporters localized in membranes of secreting mammary epithelial cells are involved in delivery of essential nutrients into milk. However, drugs and other xenobiotics may be substrates of these transporters and thus be actively secreted into milk, which may pose a health threat to breastfed infants and dairy consumers.

Aims of the thesis were to determine expressions of drug transporters in mammary gland tissue and to assess mammary cell models for studies of these proteins. Gene expressions of members of the ABC (BCRP/ABCG2, MDR1/ABCB1, MRP1/ABCC1) and SLC (OATP1A2/SLCO1A2, OCTN1/SLC22A4, OCT1/SLC22A1) families were measured in murine and bovine mammary tissue and in murine (HC11) and bovine (BME-UV) mammary epithelial cell lines.

BCRP function was assessed by transport experiments with mitoxantrone (MX) in HC11 cells. Effects of the imidazole fungicide prochloraz on transporter expression and function in HC11 and BME-UV cells were examined. Expressions of BCRP and OCT1 in murine mammary glands were increased during gestation and lactation, whereas MDR1, MRP1, OATP1A2 and OCTN1 were decreased, compared to expression in virgins. All transporters measured in mammary glands of mice were detected in bovine mammary tissue. All transporters investigated in vivo were also detected in HC11 cells, while only MDR1 and MRP1 were detected in BME-UV cells. Differentiation of HC11 cells resulted in increased BCRP protein expression, while MDR1 expression was reduced. The BCRP inhibitor elacridar reduced secretion and increased accumulation of MX in both undifferentiated and differentiated HC11 cells. An increased accumulation of MX was observed in BCRP gene silenced HC11 cells. Prochloraz treatment induced MDR1 gene expression and protein function in both differentiated HC11 and BME-UV cells, resulting in decreased accumulation of the MDR1 substrate digoxin.

In conclusion, we demonstrated that the HC11 and BME-UV mammary cell models are valuable tools for identifying substrates, inhibitors and inducers of transport proteins expressed in the mammary epithelium during lactation. The models can be used both to examine if chemical compounds are actively transported into milk and if they disrupt the normal function of transporters which may result in a disturbed delivery of essential nutrients into milk.

Keywords: active transport, mammary epithelial, HC11 cells, BME-UV cells, RNAi, milk, lactation, ABC-transporters, SLC-transporters

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To the memory of my beloved father…

Dünyada her şey için, medeniyet için, hayat için, başarı için, en hakiki mürşit bilimdir, fendir.

Mustafa Kemal Atatürk

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Jonas Tallkvist, Yagmur Yagdiran, Louise Danielsson, Agneta Oskarsson (2015). A Model of Secreting Murine Mammary Epithelial HC11 Cells Comprising Endogenous Bcrp/Abcg2 Expression and Function. *Cell Biol Toxicol*, 31 (2), pp. 111-120

II Yagmur Yagdiran, Agneta Oskarsson, Christopher H. Knight, Jonas Tallkvist. ABC- and SLC- Transporters in Murine and Bovine Mammary Epithelium - Effects of Prochloraz. (*Manuscript*).

Paper I is reproduced with the permission of the publisher.

The contribution of Yagmur Yagdiran to the papers included in this thesis was as follows:

I Participated in designing the experiments and performed the main part of the practical work, compiled and analysed the data. Contributed to the writing and publishing of the paper.

II Contributed to the planning of the work. Performed the experiments, compiled and analysed the data. Major contribution to the writing of the manuscript.

Abbreviations

ABC	ATP-binding cassette
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
BCRP	Breast cancer resistance protein (ABCG2)
BME-UV cells	Bovine mammary epithelial cell line established at the University of Vermont
cDNA	Complementary DNA
CLDs	Protein-coated cytoplasmic lipid droplets
Ct	Threshold cycle
DMSO	Dimethyl sulfoxide
EFSA	European Food Safety Authority
EGF	Epidermal growth factor
FBS	Fetal bovine serum
GF120918	Elacridar
Ham's F12	Nutrient mixture
HBSS	Hanks' Balanced Salt Solution
HC11 cells	Murine mammary epithelial cells
HUGO	Human genome organization
JAK2	Janus kinase 2
LD	Lactation day
LDH	Lactate dehydrogenase
M/P ratio	Milk to plasma ratio
MDR1	Multidrug resistance protein 1
mRNA	Messenger ribonucleic acid
MRP1	Multidrug resistance-associated protein 1
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium)

MX	Mitoxantrone
NBD	Nucleotide binding domain
NCTC 135	Nutrient mixture
OATP1A2	Solute carrier organic anion transporter family member 1A2 (SLCO1A2/OATP-A)
Oatp1a5	Solute carrier organic anion transporter family, member 1a5 (Slca1a5/Oatp3)
OCT1	solute carrier family 22 (organic cation transporter), member 1 (SLC22A1)
OCTN1	solute carrier family 22 (organic cation/zwitterion transporter), member 4 (SLC22A4)
P-gp	Permeability glycoprotein (MDR1/ABCB1)
PhIP	2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
PRL	Prolactin
PRLR	Prolactin receptor
Prochloraz	N-Propyl-N-(2-(2,4,6-trichlorphenoxy)ethyl)-1H-imidazol-1-carboxamid
RIPA buffer	Radioimmunoprecipitation assay buffer
RNAi	RNA interference
RPMI	Roswell Park Memorial Institute medium
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
SLC	Solute carrier
Stat5	Signal Transducer and Activator of Transcription 5
SYBR green	An asymmetrical cyanine dye used as nucleic stain
T3	Triiodothyronine
T4	Thyroxine
TMD	Transmembrane domain

1 Introduction

1.1 Milk and environmental toxicants

Breast milk is a valuable nutritional source for normal growth and development by providing macronutrients (sugar, fat and protein) and micronutrients (vitamins, minerals and growth factors) to the suckling infant (Pereira, 2014; Warinner *et al.*, 2014; Haug *et al.*, 2007). Nevertheless, it should be noted that milk could also be a source of chemical compounds, which may have adverse health effects in the breastfed infant (Ito & Lee, 2003; McManaman & Neville, 2003; Hallen *et al.*, 1998; Oskarsson *et al.*, 1998; Oskarsson *et al.*, 1995).

The consumption of milk and dairy products is very high and contaminants in cow's milk may cause important health problems for the population. Besides the drugs that are administered to lactating dairy cattle, these animals may also be exposed to organic pollutants, pesticides and/or mycotoxins through feed and soil. Excretion of toxicants in milk has been reported in several studies (Tornkvist *et al.*, 2011; Meucci *et al.*, 2010; Darnerud *et al.*, 2006; Schaum *et al.*, 2003). Atmospheric deposition of environmental pollutants and use of pesticides in agriculture allows grazing cows to ingest contaminated grass or hay and silage. Lipophilic properties of these toxicants lead them to distribute into fat tissues in the body including the milk fat of a lactating cow. Consequently, milk serves an efficient elimination pathway for these contaminants (Schmid *et al.*, 2003). The high consumption of milk and dairy products means that even low concentrations of toxic compounds may result in a relatively high oral intake.

1.2 Mammary gland as a secretory organ

The mammary gland development from a virgin to a milk secreting stage is stimulated by a combined action of reproductive (estrogen, progesterone and prolactin) and metabolic hormones (insulin, glucocorticoids and growth hormones) causing changes in the cellular composition of the mammary gland as well as the structural and biochemical properties of the milk synthesizing mammary epithelial cells (Neville *et al.*, 2002).

Mammary epithelial cells form a polarized single cell layer in the alveoli of lactating mammary gland (Figure 1A-B). These cells are minor units of a branching network of ducts and responsible for the secretion of several milk components into the lumen where the milk is stored (Figure 1B) (Richert *et al.*, 2000). Myoepithelial cells discontinuously surround the alveoli and establish direct contact with the basement membrane (Figure 1B). Milk ejection through the ducts is accomplished by contraction of myoepithelial cells by the action of oxytocin which is released into the systemic circulation as a result of nipple stimulation. The alveoli of the lactating gland are also surrounded with a vascularized connective tissue stroma that is composed of lipid-depleted adipocytes and fibroblasts (Figure 1B) (McManaman & Neville, 2003; Richert *et al.*, 2000). At the end of lactation, mammary gland undergoes involution where this secretary organ returns to the pre-pregnant stage (McManaman & Neville, 2003; Richert *et al.*, 2000).

1.3 Passive and active transport by several transport pathways

1.3.1 Secretion routes

Well-organized mechanisms act in concert to deliver essential nutrients from the blood side across mammary epithelial cells followed by its release into milk, and non-nutritional compounds may utilize same mechanisms to enter milk. Thus, different secretory transport pathways may be responsible for the presence of drugs and other xenobiotics in milk.

Endogenous substances like major milk proteins, lactose, oligosaccharides, citrate, phosphate and calcium are transported to milk across the apical membranes of alveolar cells via the exocytotic pathway (Figure 1C, III) (McManaman & Neville, 2003). Milk fat globules originating from triglyceride and cholesteryl esters are released as protein-coated cytoplasmic lipid droplets and secreted into milk via the lipid secretion pathway (Figure 1C, II) which is a process unique to mammary epithelial cells (Russell *et al.*, 2007) Macromolecular substances such as immunoglobulins, albumin and transferrin enter milk via transcytotic pathway (Figure 1C, V). Active transport requires

the presence of specific transport proteins in both apical and basolateral membranes of epithelial cells. Small molecules including ions, amino acids and glucose are actively transported into milk via this protein-mediated pathway (Figure 1C, I). Toxic compounds may also cross the lactating mammary epithelium via active transport mechanisms (protein-mediated pathway, I) or via passive transcellular diffusion of lipohilic compounds (transcytotic pathway, V). If tight junctions are affected xenobiotics may enter milk passively (paracellular pathway, IV).

The present study will focus on the details of active transport and the transporters during lactation (Figure 1C, protein-mediated pathway, I). Many of the transport proteins comprising this pathway feature broad substrate specificities and may thus actively transport both endogenous and exogenous compounds across the lactating mammary epithelium.

1.3.2 Factors influencing chemical transfer into milk

Hence, there are two basic mechanisms for secretion of toxic compounds into milk. In case of passive diffusion uncharged and lipophilic chemicals are secreted into milk until the concentration of the residues reaches equilibrium between plasma and milk. The amount of residues present in milk can be predicted by molecular size of the chemical, lipophilicity, plasma protein binding and pKa (Fleishaker, 2003; Agatonovic-Kustrin *et al.*, 2002).

Calculation of the ratio between the concentrations of chemicals in milk and in plasma (M/P ratio) over time is used to assess participation of passive or active processes. In the case of passive diffusion the M/P ratio is ≤ 1 and in case of active transport M/P ratio is > 1 (Fleishaker, 2003; Ito & Lee, 2003; Gerk *et al.*, 2001).

1.4 Transporters

A large number of membrane transport proteins have been characterized. Two major classes are involved in the transfer of drugs and xenobiotics classified as the ATP-binding cassette (ABC-) and solute carrier (SLC-) transporters. Members of these families are expressed in the apical or basolateral membrane of polarized epithelial cells in the intestine, liver, kidney, blood brain barrier, placenta as well as mammary gland and play an important role in chemical absorption, distribution and elimination in the body (Russel, 2010; Sai & Tsuji, 2004; Ito & Alcorn, 2003).

Transport proteins belonging to both the ABC- and SLC- superfamilies are expressed at various levels depending on lactation stage of the mammary gland (Gilchrist & Alcorn, 2010; Jonker *et al.*, 2005; Alcorn *et al.*, 2002). This

altered expression allows some of these transporters to deliver vitamins, fatty acids, sterols, porphyrins and amino acids to the nursed offspring via milk (Vlaming *et al.*, 2009; van Herwaarden *et al.*, 2007; Kwok *et al.*, 2006). The transporters have substrate specificity not only for nutrients but also for drugs and xenobiotics which may be actively transported resulting in high concentrations in milk (Figure 1C) (Ito *et al.*, 2014; Kindla *et al.*, 2011; Gilchrist & Alcorn, 2010; Kwok *et al.*, 2006; Jonker *et al.*, 2005; Ito & Alcorn, 2003).

1.4.1 ATP-binding cassette (ABC) transporters

ABC transporters are membrane proteins consisting of seven subfamilies named A to G (Szakacs *et al.*, 2008). Their existence and conserved structure across different organisms emphasize the fact that these transporters are fundamental for all living systems (Jones & George, 2004). ABC transporters function as efflux pumps and are involved in the excretion of chemicals across the membranes of epithelial cells against a concentration gradient into the bloodstream or a lumen using ATP hydrolysis as energy source (Russel, 2010; Couture *et al.*, 2006). ABC transporters generally have the similar conserved structure that is composed of transmembrane domains (TMDs) and nucleotide-binding domains (NBDs) (Szakacs *et al.*, 2008; Chan *et al.*, 2004; Jones & George, 2004). Three subfamilies of this family are particularly involved in transport of drugs and xenobiotics, which are known as *ABCB*, *ABCC* and *ABCG* families.

Breast cancer resistance protein (BCRP/ABCG2)

BCRP belongs to the G subfamily of the ABC family. The gene was first cloned from a human breast carcinoma cell line in 1998 (Doyle *et al.*, 1998). BCRP is a half transporter protein comprised of one NBD and one TMD with six transmembrane α-helices. Thus, BCRP is thought to function as a homodimer (Jani *et al.*, 2014; Staud & Pavek, 2005; Chan *et al.*, 2004). BCRP is a 72-kDa protein with 665 amino acids (Staud & Pavek, 2005) detected in epithelial of numerous normal (non-tumorigenic) tissues such as colon, liver, placenta, intestine, mammary glands and many others (Fetsch *et al.*, 2006; Alcorn *et al.*, 2002; Maliepaard *et al.*, 2001). From the physiological point of view, apical localization of BCRP in the polarized cells exhibits a general protective role by reducing the levels of drugs and xenobiotics in the cells by active efflux of its substrates (Maliepaard *et al.*, 2001).

Expression of BCRP is induced in the apical membrane of mammary epithelial cells in mammary glands of various species during lactation (Jonker *et al.*, 2005). Although, BCRP generally has a protective function in other

tissues, the reason for its role in the mammary gland remains unclear. However, BCRP has substrate specificity to a number of vitamins including vitamin K3 (Shukla *et al.*, 2007), riboflavin (van Herwaarden *et al.*, 2007) and folic acid (Assaraf, 2006). Apical expression of BCRP along with the induced expression in lactating mammary epithelial cells may suggest a physiological role for the secretion of these nutrients into milk during lactation (Lindner *et al.*, 2013; van Herwaarden *et al.*, 2007; Alcorn *et al.*, 2002).

Unfortunately, several drugs and toxins, including topotecan, 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) (Jonker *et al.*, 2005), mitoxantrone (Imai *et al.*, 2003; Litman *et al.*, 2000), nitrofurantonin (Merino *et al.*, 2005), ciprofloxacin (Merino *et al.*, 2006), and enrofloxacin (Pulido *et al.*, 2006), have been identified as BCRP substrates which are transported and concentrated in milk by this efflux protein.

Prochloraz (N-propyl-N-[2-(2,4,6-trichlorophenoxy)ethyl]-1H-imidazole-1-carboxamide) is an imidazole fungicide used in agriculture and horticulture that may cause a general exposure through consumption of fruit and vegetables (EFSA, 2011). Prochloraz is an endocrine disruptor with various modes of action (Ohlsson *et al.*, 2009; Vinggaard *et al.*, 2006). Treatment of primary bovine mammary epithelial cells with prochloraz resulted in increased BCRP expression through AhR activation (Halwachs *et al.*, 2013).

Multi drug resistance protein 1 (MDR1/ABCB1)

MDR1 (also known as Permeability-glycoprotein or P-gp) was the first cloned drug carrier from colchicine-resistant Chinese hamster ovary cells (Juliano & Ling, 1976) and is the best characterized protein of the ABC transporter family (Silva *et al.*, 2015). The gene encodes a 170-kDa single polypeptide with 1280 amino acids (Hoffmann & Kroemer, 2004; Leonessa & Clarke, 2003). Similar to other ABC family members, MDR1 consists of two TMDs each containing six transmembrane helices and two cytoplasmic NBDs (Jones & George, 2004).

MDR1 is present with a high expression at the apical surface of the epithelial cells in liver, colon, kidney proximal tubules and pancreatic ductules. The tissue localization as an efflux transporter in excretory organs, suggest that MDR1 plays a role in the protection of the organs by effluxing metabolites and xenobiotics out of the epithelial cells (Silva *et al.*, 2015).

Beside these tissues, MDR1 is expressed in mammary glands of mouse, rat and human although with lower abundance during lactation (Gilchrist & Alcorn, 2010; Jonker *et al.*, 2005; Alcorn *et al.*, 2002). MDR1 has broad substrate specificity to hydrophobic neutral or positively charged molecules

including anticancer chemotherapeutic reagents and xenobiotics (Ito & Alcorn, 2003). The physiological role for the decline in expression of MDR1 in the apical membranes of secreting mammary epithelial cells remains obscure but may reflect an adaptive response to prevent efflux of xenobiotics or drugs into milk in order to protect the nursing neonate from exposure (Alcorn *et al.*, 2002).

Multidrug resistance-associated protein 1 (MRP1/ABCC1)

MRP1 is one of nine members of ATP-binding cassette C subfamily. It was first cloned from a multidrug resistant human small cell lung cancer cell line (H69R) that does not express MDR1 (Bakos & Homolya, 2007; Chan *et al.*, 2004; Cole *et al.*, 1992). MRP1 gene encodes a 190-kDa protein comprising 1531 amino acids. It features three TMDs instead of two and 17 transmembrane helices together with two NBDs (Gradhand & Kim, 2008).

MRP1 is an efflux pump and notably expressed on the basolateral membrane of sarcolemma cells in heart, bronchial epithelium cells in lungs, Leydig and Sertoli cells in testes, crypt cells in intestine, urinary collecting ducts and glomeruli in kidney but also on the apical membrane of endothelial cells in placenta and capillary endothelial cells in brain (Klaassen & Aleksunes, 2010; Leslie *et al.*, 2005). Substrates of MRP1 include amphipathic compounds and glutathione S-conjugates, oxidized glutathione, leukotriene C4 and several cancer chemotherapeutics (Chan *et al.*, 2004). Although the subcellular localization of MRP1 has not yet been verified, gene and protein expressions have been shown to be down regulated during lactation in human mammary epithelial cells as well as in mammary glands of mice and rats (Gilchrist & Alcorn, 2010; Jonker *et al.*, 2005; Alcorn *et al.*, 2002).

1.4.2 Solute Carrier (SLC) Transporters

The solute carrier transporter family is the largest group of transporters with 298 members in 43 subfamilies based on HUGO assembly sequences. The members are divided based on their function and different biochemical properties (Fredriksson *et al.*, 2008). As a result, they can function as coupled transporters, exchangers/antiporters related with cellular sodium gradient and as passive or active transporters (Fredriksson *et al.*, 2008). Unlike the ATP family, members of the SLC family use secondary and tertiary active transport typically in the uptake (influx) or bidirectional transport of chemicals across biological membranes (Klaassen & Aleksunes, 2010). To assure the driving force by secondary active transport against a diffusion gradient, there should be a connection to the symport or antiport of inorganic or organic ions. Most of

the proteins in the family generally have the similar structure with 12 putative transmembrane domains and have molecular sizes between 50-100-kDa (Russel, 2010). Localization of these transporters can be either apical or basolateral depending on the tissue and cell type (Roth *et al.*, 2012).

In the present study we will focus on some representative transporters from three different subfamilies including the organic anion organic polypeptide (SLCO), organic cation transporter (SLC47) and organic cation/carnitine transporter (SLC22) families.

Solute carrier organic anion transporter family member 1A2 (OATP1A2/SLCO1A2)

OATP1A2 is a member of SLCO family and it was isolated by hybridization screening from human liver as the first human superfamily member (Hagenbuch & Meier, 2003). Radiation hybrid analysis and fluorescent in situ hybridization have demonstrated that the human OATP1A2 gene [previously called OATP-A (Hagenbuch & Meier, 2004)] is localized to chromosome 12. These studies also showed that OATP1A2 is the human orthologue of mouse Oatp1a5 [previously called Oatp3 (Hagenbuch & Meier, 2004)] High degree of identity (72%) supported this argument when OATP1A2 and Oatp1a5 amino acid sequences were aligned (Walters *et al.*, 2000).

OATP1A2 is an influx transporter that functions in a Na+ independent manner. This transporter has broad substrate specificity particularly to anionic compounds (Arakawa *et al.*, 2012). Human OATP1A2 is apically localized on cholangiocytes in liver, enterocytes in small intestine, and distal tubules in kidney. Mouse and rat Oatp1a5 also have apical localization in epithelial cells of choroid plexus, endothelial cells of and brain and enterocytes of jejunum, respectively (Klaassen & Aleksunes, 2010).

Expression of OATP1A2 (OATP-A) was detected in human mammary epithelial cells and transcript levels demonstrated to be upregulated during lactation (Alcorn *et al.*, 2002). OATP1A2 (Oatp1a5) has a significant role in non-tumorigenic tissues by mediating the transport of endogenous substrates such as conjugated and free bile salts, thyroid hormones (T3 and T4), steroids and steroid conjugates. Similar to other transporters OATP1A2 has also substrate specificity to exogenous compounds and drugs including erythromycin and imatinib (Liu & Li, 2014; Kalliokoski & Niemi, 2009; Ito & Alcorn, 2003). The physiological role for OATP1A2 induction in the lactating mammary gland remains unclear but may suggest a contribution to organic anion transfer in mammary epithelium resulting in an accumulation of this type of compounds in milk.

Organic cation transporter 1 (OCT1/SLC22A1)

OCT1 was found as a first member of organic cation transporter family that was cloned from a rat kidney cDNA library (Grundemann *et al.*, 1994). Shortly after, its mouse and human orthologues were also cloned (Klaassen & Aleksunes, 2010). Human OCT1 mRNA is predominantly expressed in liver compared to other organs whereas high abundances for the murine orthologue Oct1 was observed in both liver and kidneys (Koepsell, 2013; Klaassen & Aleksunes, 2010; Koepsell & Endou, 2004). OCT1 is also induced during lactation in mammary glands of mouse, rat and human (Ito *et al.*, 2014; Gilchrist & Alcorn, 2010; Alcorn *et al.*, 2002). Cellular localization of the protein can differ depending on the tissue and species. Oct1 proteins have been reported to be localized to the basolateral membrane of proximal tubules in rat kidney while others have reported localization of OCT1 in the apical membranes of proximal and distal tubules in humans (Koepsell, 2013; Klaassen & Aleksunes, 2010; Koepsell & Endou, 2004). OCT1 mediates transport of organic cations that are positively charged at physiological pH. This transport functions in an electrogenic manner that is independent from sodium. The substrate list for OCT1 is wide and include many endogenous compounds such as neuromodulators cyclo (His-Pro), prostaglandins, salsolinol and metabolites like putrescine and agmatine and drugs such as antiparkinsonians, antidiabetics, some carcinogen toxins like aflatoxin B1 and ethidiumbromide (Koepsell, 2013; Jonker & Schinkel, 2004; Sai & Tsuji, 2004).

In general, OCT1 has roles in the hepatic excretion of cationic drugs, reabsorption of ultra-filtrated cations in kidney, the absoption of drugs in lungs and also passage of endogenous substrates of drugs across blood-brain barrier (Koepsell, 2013).

The exact membrane localization of OCT1 in mammary gland remains to be elucidated. Therefore, it is difficult to make interpretation about its physiological roles in lactating mammary gland. However, secretion of important nutrients into milk such as thiamine (vitamin B1) may suggest one of the possible role of OCT1 (Ito *et al.*, 2014). Furthermore, prostaglandins which are substrates of OCT1 are found in human milk that may be in relation to the protective role of milk lipids (Lucas & Mitchell, 1980). In addition, cooperative vectorial transport of cimetidine and acyclovir into milk, which are substrates for both Bcrp and Oct1, suggested that Oct1 probably expressed on the basolateral side of the membrane in mouse mammary epithelial cells. Oct1 exhibits substrate uptake from blood into the cell while Bcrp permits substrate secretion from cell into the milk (Ito *et al.*, 2014).

Organic cation transporter novel protein type1 (OCTN1/SLC22A4)

OCTN1 is encoded by SLC22A4 gene, which was cloned from human fetal kidney (Tamai *et al.*, 1997). OCTN1 is expressed in different tissues such as intestine, liver, kidney, brain, heart, skeletal muscle and mammary gland of mouse, rat and human (Koepsell, 2013; Gilchrist & Alcorn, 2010; Alcorn *et al.*, 2002). Immunohistochemical studies revealed that Octn1 is predominantly localized in the blood vessel endothelium and in the apical membranes of secretory mammary cells in rat mammary glands (Lamhonwah *et al.*, 2011). A recent study has also demonstrated that OCTN1 is localized within the mitochondrial cells suggesting a possible mechanism required for carnitine transport into the organelle (Lamhonwah & Tein, 2006).

OCTN1 has been shown as multi-specific, bidirectional, pH dependent transporter (Yabuuchi *et al.*, 1999) therefore it may undertake different roles in the plasma membranes as cation exchanger, as sodium dependent or independent transporter for zwitterions and as organic cation/proton exchanger that exhibits crucial functions for the system (Koepsell, 2013). Similar to other transporters reviewed above, OCTN1 also has substrate specificity to endogenous compounds as well as drugs and xenobiotics. Among all these, the most prominent substrates are zwitterions that include L-carnitine. It is a small water-soluble compound has 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 (Lamhonwah *et al.*, 2011). Octn1/OCTN1 gene and protein expression have been demonstrated to be up regulated during lactation in mammary gland of rats and in human mammary epithelial cells (Lamhonwah *et al.*, 2011; Gilchrist & Alcorn, 2010; Ling & Alcorn, 2010; Alcorn *et al.*, 2002). L-carnitine, which is essential for early brain growth and development of the physiologically immature infant, is not synthesized endogenously and delivery is maintained by protein-mediated transport into milk.

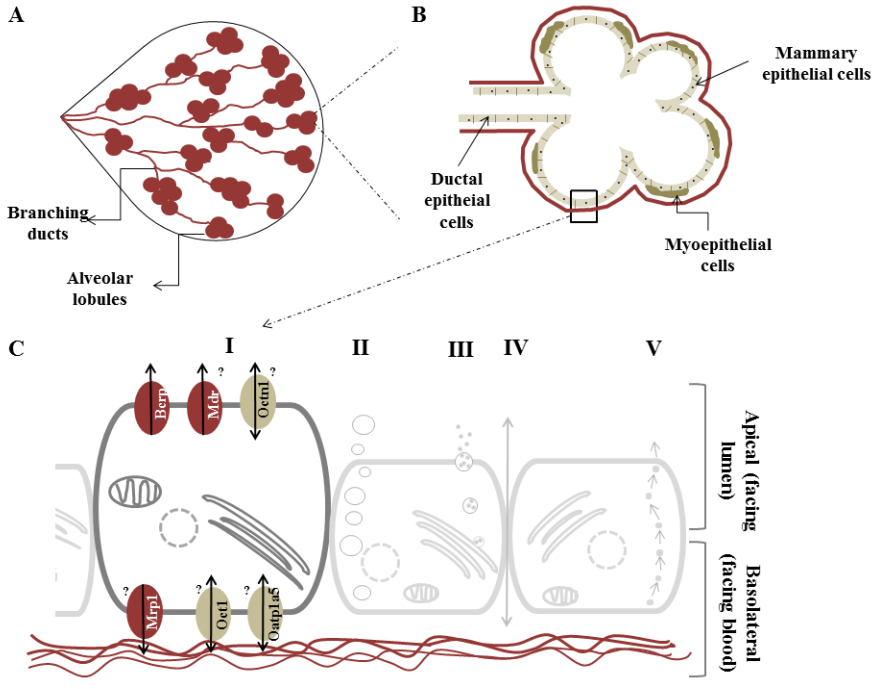

Figure 1. A schematic illustration of a mammary gland at lactating stage (A). Mammary alveolus consists of different cell types. Mammary epithelial cells are surrounded by myoepithelial cells that play an important role in ejection of milk into the ductal lumen (B). Transport pathways that occur in the mammary epithelial cells. *I: Protein-mediated transport pathway; II: Lipid secretion pathway; III: Exocytotic pathway; IV: Paracellular pathway; V: Transcytotic pathway (C). *Active transport of chemical compounds via the protein mediated pathway is carried out by transport proteins that belong to ABC and SLC superfamilies. Cellular localizations of the transporters shown in the figure with (?) are predictions based on localizations previously demonstrated in other tissues.

1.5 Murine (HC11) and bovine (BME-UV) mammary epithelial cell lines as in vitro models

Polarized cell lines without recombinant genes and proteins allow us to study endogenous transport mechanisms. Therefore, the cell lines chosen for studying these processes must exhibit functional and morphological properties similar to in vivo cell layers in order to make correlations. Consequently, these models can be applied as tools to gain knowledge regarding function of transport mechanisms where the findings may be extrapolated to humans.

HC11 cells are derived from mammary glands of BALB/c mice during mid-pregnancy (Danielson *et al.*, 1984). This cell line is the first clone of non-tumorigenic COMMA-1D cells that take on a secreting phenotype upon prolactin treatment resembling mammary epithelial cells in the lactating mammary gland. β-casein induction is accomplished in the presence of lactogenic hormones especially by prolactin without any requirement for an extracellular matrix or co-cultivation with adipocytes and/or fibroblasts (Ball *et al.*, 1988).

BME-UV cells are non-tumorigenic mammary epithelial cells originating from mammary gland tissue of a lactating and pregnant Holstein cow. Optimal conditions for casein synthesis for the BME-UV cells have not yet been established. However, there is evidence that BME-UV cells are producing low levels of α_{s1}-casein and k-casein and displaying some of the important functions of mammary epithelial cells (Zavizion *et al.*, 1996).

Morphological and functional changes in mammary epithelial cells in vitro are regulated with signalling pathways (Groner & Gouilleux, 1995). These pathways are synergistically regulated by different hormones including epidermal growth factor (EGF), insulin, prolactin (PRL) and glucocorticoids (Pauloin & Chanat, 2012; Shan *et al.*, 2008). In the presence of insulin and EGF, mammary epithelial cells grown to confluency, become competent and ready to respond to lactogenic hormones (Shan *et al.*, 2008). The initial step in the mechanism of action of PRL starts when it binds to the cell surface receptor, prolactin receptor (PRLR). This coupling causes ligand-induced dimerization and leads to phosphorylation and activation of JAK2 (Janus) kinases. Subsequently, this induces phosphorylation of the tyrosine residues in the C-terminal tail of the prolactin receptor. As a result, it creates specific domains for proteins like signal transducer and activator of transcription 5 protein (Stat5), which is capable of, translocate to nucleus. Transcription of genes that code for milk proteins starts when activated Stat5 dimers bind to certain specific sites on their promoter region (Bole-Feysot *et al.*, 1998; Groner & Gouilleux, 1995).

The synergistic effect of glucocorticoids with insulin and prolactin regarding differentiation should be also taken into consideration (Desrivieres *et al.*, 2007). Physical interaction of glucocorticoid receptor with Stat 5 upon glucocorticoid binding assists the ability of Stat5 to activate β-casein transcription (Desrivieres *et al.*, 2007). In addition, it has been shown that induction of genes that code milk proteins failed when one of these hormones eliminated (Pauloin & Chanat, 2012; Desrivieres *et al.*, 2007; Stocklin *et al.*, 1996).

2 Aims of the thesis

The overall aims of thesis were to determine expressions of ABC- and SLC-transporters in mammary gland tissue and to assess whether the HC11 and BME-UV mammary cell models can be applied to determine substrates, inducers and inhibitors of transporters expressed in the mammary epithelium during lactation.

More specific aims were to:

1. Investigate gene expressions of endogenous Bcrp, Mdr1, Mrp1, Oatp1a5, Octn1 and Oct1 in mammary glands of mice in virgins and at various gestation and lactation stages and BCRP, MDR1, MRP1, OATP1A2 and OCTN1 in mammary glands of lactating cows.

2. Examine gene expressions and function of transporters in HC11 cells with a secretion phenotype and BME-UV cells derived from a lactating cow.

3. Assess the effect of prochloraz on gene expression and function of transporters in the HC11 and BME-UV cell models.

3 Materials and Methods

The materials and methods used in this thesis are described and presented in detail in each paper. The following is a short overview of the methods compiled from paper I and paper II.

3.1 Chemicals

All chemicals used were of analytical grade and purchased from regular commercial sources. Detailed information on chemicals and the commercial sources of reagents are presented in the papers.

3.2 Experimental models

3.2.1 Mice

Mammary gland tissues of mice were either used to test the specificity of primers for the real-time RT-PCR with cellular RNA and to examine mRNA expression levels of transporters at different lactation stages.

Litter sizes were normalized to ten on lactation day (LD) 1 and the dams with litters were kept in individual cages (the day of parturition = LD 0). On these respective days of pregnancy (gestational day 13 and18), lactation (day 2 and 9), involution (weaning day 2) or virgin animals were killed by cervical dislocation and mammary glands were rapidly dissected and stored in RNAlater solution at -70°C until total RNA extraction. All animal experiments were approved by a Local Ethics Committee of Animal Research (permit no. 2012-15-2934-00587).

3.2.2 The HC11 murine mammary epithelial cells (papers I and II)

HC11 cells were maintained in RPMI 1640 medium containing insulin, fetal bovine serum (FBS), epidermal growth factor (EGF) and gentamycin in an

atmosphere of 95% air and 5% CO_2 in 95% relative humidity. The cell culture medium was changed every 2-3 days and cells sub-cultured when they reached 80-90% confluency. To induce lactogenic differentiation, medium was replaced with differentiation medium supplemented with prolactin and hydrocortisone (EGF and FBS free) and incubated for additional 72 h.

3.2.3 The BME-UV bovine mammary epithelial cells (paper II)

Cells were maintained in sterile filtered RPMI 1640 medium supplemented with FBS, Ham's F12, NCTC 135, lactose, lactalbumin hydrolysate, GSH, L ascorbic acid, hydrocortisone, insulin and gentamycin in polycarbonate flasks at 37°C in a humidified 5% CO_2 incubator until confluence as described (Zavizion *et al.*, 1996). Prolactin was added to the cells 72 h prior to isolation of RNA for gene expression measurements or transport experiments as described (Liu *et al.*, 2006).

3.3 Experimental Techniques

3.3.1 Cellular organization of HC11 cells (paper I)

Cells were seeded on a chamber slide and grown until confluence. Chambers covering half of the slide were used for undifferentiated HC11 cells (controls) while the cells in other half were differentiated as described above. Subsequently, cells were fixed with refrigerated acetone and stained with hematoxylin in order to examine cellular organization under microscope.

3.3.2 Histological characterization of mammary gland tissues of mice

Samples of the mammary glands for histology were fixed in 4% paraformaldehyde and dehydrated in ethanol saline buffer. Tissues were imbedded in paraffin and sections were stained with hematoxylin and eosin.

3.3.3 Cytotoxicity

Cytotoxicity was measured by MTS test that was carried out according to the instructions of the manufacturer (Promega).

3.3.4 Gene expression

The quantitative gene expression of the murine and bovine β-casein, Bcrp, Mdr1, Mrp1, Oatp1A2/1a5, Octn1, Oct1 and murine Cyclophilin B was measured by real time RT-PCR on a RotorGene 3000 using the One-tube QuantiTect™ SYBR®Green RT-PCR kit according to the manufacturer's instructions (for details, see papers I and II). The primers used, which were specific for both murine and bovine mRNA, are presented in Table 1 (papers I

and II). Gene expressions were normalized to total RNA and relative quantifications of normalized gene expressions were performed by comparing the threshold cycles (Ct) of untreated and treated samples according to the $2^{-\Delta Ct}$ method (Livak & Schmittgen, 2001). Fold differences were calculated setting untreated controls to one.

3.3.5 Protein expression

Protein expressions of drug transporters in the murine mammary epithelial cells (HC11) were quantified by Western blot. HC11 cells and mammary gland tissues were homogenized in RIPA-lysis buffer and total protein concentrations were quantified using the bicinchoninic acid (BCA) protein assay. Cellular proteins were separated by SDS-PAGE and blotted on to nitrocellulose membranes prior to blocking and antibody (Bcrp and Mdr1) hybridizations (for detail, see paper I). Intensities of obtained bands were quantified by the OptiQuant software using a ChemiDoc instrument (BioRad). In the analyses expression of target proteins was normalized to protein expression of tubulin.

3.3.6 Gene silencing by small interfering RNAs (paper I)

RNA interference (RNAi) is a process where target genes are silenced in a sequence specific manner with short interfering RNAs (siRNA) (Dykxhoorn *et al.*, 2003).

We first optimized transfection efficiency conditions in HC11 cells by testing various cell densities and dilutions of transfection reagents. Optimization was accomplished using the housekeeping gene cyclophilin B. Following gene silencing, efficient siRNA transfection was verified by quantitative real-time RT-PCR with specific primers to cyclophilin B. Using the same protocol with Bcrp siRNA-duplexes Bcrp-transcript levels were reduced to the same extent as cyclophilin B.

3.3.7 Transport Experiments (papers I and II)

For both accumulation and secretion studies, HC11 and BME-UV cells were seeded in 12 well plates, cultured and treated as described above. Mitoxantrone (MX) or digoxin were used to examine the function of BCRP or MDR1 respectively in the HC11 and BME-UV using ^{3}H-MX or ^{3}H-digoxin as tracers. Both accumulation and secretion of MX or digoxin were normalized to total cellular protein of the HC11 and BME-UV cells (for details, see papers I and II).

3.4 Statistics

Non-parametric methods; Kruskal-Wallis and Mann-Whitney Rank-Sum tests were used. Data was first analyzed by the Kruskal-Wallis test to detect statistical significant differences between experimental groups. Subsequently, Mann-Whitney Rank-sum test was applied to demonstrate statistically significant differences between pairs of groups. The level of significance was set at $p \leq 0.05$. All statistical analyses were performed in Statview or MiniTab.

4 Results and Discussion

4.1 Histology

4.1.1 Mammary gland development in mouse (paper II)

Histological changes in mammary gland of mouse throughout different stages are shown in Figure 2B. It is a brief presentation of morphological changes that occur during mammary gland development. Prior to pregnancy, mammary gland consists mainly of adipose tissue (V). During gestation days 13-18, alveolar lobules start to develop and partly fill the fat pad (G13 and G18). The epithelial cells are able to produce milk proteins and lipids at gestation day 18 (arrow, G18). Alveolar lobules turn into secretory structures that are surrounded by adipocytes at the beginning of lactation (L2). During peak lactation (L9) the mammary gland consists mainly of alveolar structures with large and milk filled lumina. At the end of lactation, a remodelling of the mammary gland occurs in a process called involution comprising a degeneration of alveolar cells and an increase in fat content, typical of non-lactating mammary glands observed prior to pregnancy (W2).

Knowledge about mammary gland development may provide insight into its histological characteristics during lactation. Thus, morphological differences between virgin, pregnant and lactating mammary gland tissues can be used as a marker for differentiation of cultured mammary epithelial cells.

4.1.2 Cellular organization of HC11 cells (paper I)

Lactogenic differentiation of the HC11 cells by prolactin and glucocorticoids resulted in the following morphological changes (Figure 2A). Undifferentiated HC11 cells were organized as a monolayer (Figure 2A, left), while alveolar-like structures including lumina appeared in the differentiated cells resembling the pattern that is observed in the mammary glands in vivo during lactation (Figure 2B) (Richert *et al.*, 2000; Burgoyne & Duncan, 1998).

4.2 Induction of β-casein gene expression (papers I and II)

Beside the changes in cellular organization by lactogenic stimulation β-casein gene expression was also induced in the HC11 cells. The relative β -casein gene expression was significantly increased 2-fold in the differentiated as compared to the undifferentiated HC11 cells (Figure 2C, left). It has previously been demonstrated that β -casein responds to prolactin in HC11 cells (Ball *et al.*, 1988) and that synthesis occurs in rodent mammary epithelial cells in vivo and that the abundance of this milk protein increases during gestation and peaks at mid-lactation (McManaman & Neville, 2003; Richert *et al.*, 2000; Burgoyne & Duncan, 1998; Robinson *et al.*, 1995). Our results once more demonstrated a gradual increase in β-casein expression through gestation days 13 and 18, which continued at lactation days 2 and 9 (Figure 2C right). Compared to the highest level observed at lactation day 9 the expression was reduced at weaning day 2 ($p=0.05$) (Figure 2C, right). Thus, reorganization of HC11 cells into alveolar-resembling formations in combination with an increased β -casein gene expression by lactogenic stimulation appears to be reliable markers of a secreting phenotype.

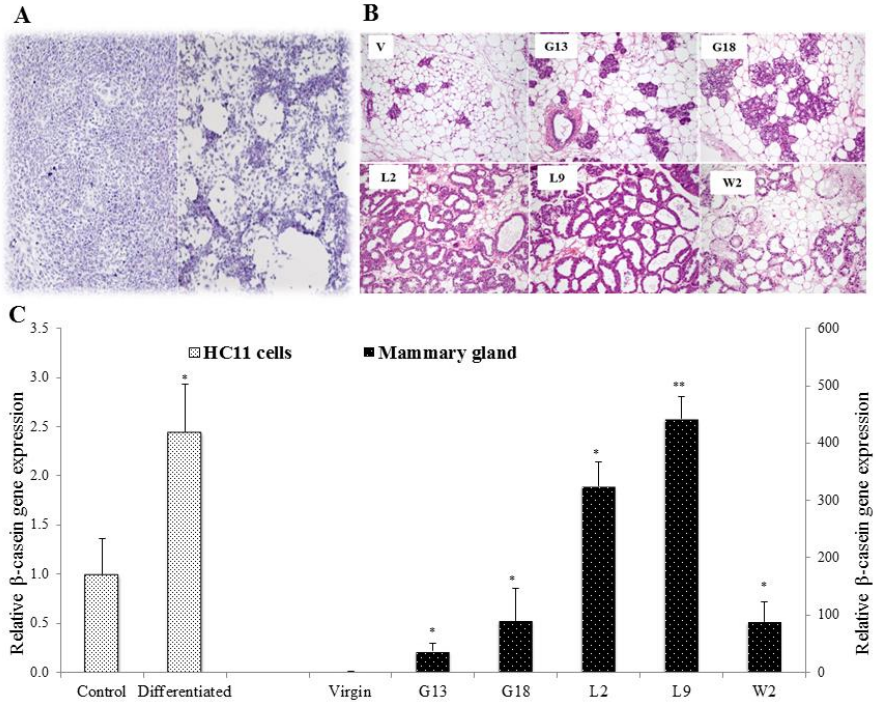

Figure 2. Cellular organization of undifferentiated (left) and differentiated (right) HC11 cells (A). Histological characterization and progression of mammary gland differentiation in virgin mice (V), during pregnancy (G13 and G18), lactation (L2 and L9) and involution (W2) (B). All sections have been stained with hematoxylin and eosin. Magnification 20X. β-casein expression in undifferentiated and differentiated murine (HC11) mammary epithelial cells (left) and murine mammary gland at various stages (right) (C). Statistically significant differences as compared to undifferentiated controls or virgins *p ≤0.05; **p≤0.01.

4.3 Expressions of transporters (papers I and II)

4.3.1 In vivo (mammary glands of mouse and cow)

Messenger RNA expression levels of Bcrp, Mdr1, Mrp1, Oatp1a5, Octn1 and Oct1 in mammary gland tissues were measured at different stages of gestation and lactation of mice by real time RT-PCR (paper II). Expressions were normalized to mRNA levels in virgins and presented as fold differences in Table 1 (for details, see paper II).

Our results revealed that all transporters sought were present in the mouse mammary glands with different expression patterns. Bcrp expression in the mice followed a similar pattern as β-casein expression with a continuous increase during gestation and lactation and a relative reduction at weaning day

2 compared to lactation day 9 (p=0.05) (Table 1). A gradual decrease in Mdr1 expression was detected in the mammary gland tissues of mice through gestation days 13 and 18 which continued at lactation days 2 and 9 (Table 1). At weaning day 2 Mdr1 expression was increased as compared to lactation day 9 (p=0.05) (Table 1). Gene expression of Mrp1 followed a similar pattern as Mdr1 with a successive reduction in transcript levels through gestation and lactation and a relative increase at weaning day 2 (Figure 1d). Oatp1a5 gene expression started to decrease in mouse mammary gland tissues at late gestation and was lowest at lactation day 9 (Table 1). Similar to Oatp1a5, mRNA levels of Octn1 mRNA decreased continuously through gestation, lactation and weaning. Oct1 expression was increased at gestation and lactation and reduced at weaning day 2 (Table 1).

Table 1. *Fold differences in transporter mRNA levels in mammary glands of mice compared to virgin (mean of 3-4 mice).*

Transporter Gene	Virgin	G13	G18	L2	L9	W2
Bcrp	1	3.47	14.29	23.5	31.01	3.23
Mdr1	1	0.40	0.23	0.09	0.07	0.18
Mrp1	1	0.41	0.35	0.08	0.05	0.20
Oatp1a5	1	1.41	0.37	0.04	0.01	0.02
Octn1	1	0.51	0.14	0.04	0.03	0.10
Oct1	1	0.98	1.42	1.69	1.56	0.32

In lactating human mammary epithelial cells OATP1A2 and OCTN1 are upregulated (Alcorn *et al.*, 2002). Our results showed that both these SLC transporters were expressed in murine mammary epithelial cells but were down-regulated in the lactating mammary glands of mice. The reason for this discrepancy in expressions is not known. However, possible explanations may involve species differences or the fact that mammary gland tissue is comprised not only of epithelial cells but also of other cell types such as myoepithelial cells and ductal cells which may contribute to this result. Supporting this hypothesis, our results with the cultured murine mammary epithelial HC11 cells showed that mRNA levels of Oatp1a5 were increased and Octn1 transcript levels not reduced by prolactin induced differentiation. In line with previous reports our results demonstrated that both Bcrp and Oct1 were most abundant during peak lactation as assessed by the peak in β -casein expression, whereas Mdr1 and Mrp1 expressions were reduced at this stage (Table 1) (Ito

et al., 2014; Shi *et al.*, 2014; Gilchrist & Alcorn, 2010; Jonker *et al.*, 2005; Alcorn *et al.*, 2002; Robinson *et al.*, 1995).

In bovine mammary gland tissue β-casein, BCRP, MDR1, MRP1, OATP1A2 and OCTN1 gene expressions were detected (data not shown). A limited number of cows were analysed and no data on expression related to various lactation stages is available. However, the results showed that the bovine specific primers and RT-PCR method is functional.

4.3.2 In vitro (murine HC11 and bovine BME-UV mammary epithelial cells)

In papers I and II, gene expressions of Bcrp, Mdr1, Mrp1, Oatp1a5, Octn1 and Oct1 were identified in both undifferentiated and differentiated HC11 cells. Statistically significant up-regulation in Oatp1a5 gene expression was observed in the differentiated HC11 cells as compared to the undifferentiated controls (Figure 3, left). Mdr1 gene expression was statistically significantly reduced in the differentiated HC11 cells as compared to the undifferentiated controls (Figure 3, left). No significant difference due to differentiation was observed in Bcrp, Mrp1, Octn1 and Oct1 gene expressions (Figure 3, left). The reason for the lack of induction of Bcrp mRNA in the HC11 cells by lactogenic stimulation is unknown. However, one possible explanation may be that the HC11 cells originally derive from mammary tissue of BALB/c mice during gestation when Bcrp transcript levels are induced to a stage where no further increases in gene expression occur (Jonker *et al.*, 2005; Danielson *et al.*, 1984). Among the genes examined only MDR1 and MRP1 were detected in the BME-UV cells (Figure 3, right). No difference was observed in MDR1 or MRP1 gene expressions after prolactin treatment of the cells (Figure 3, right).

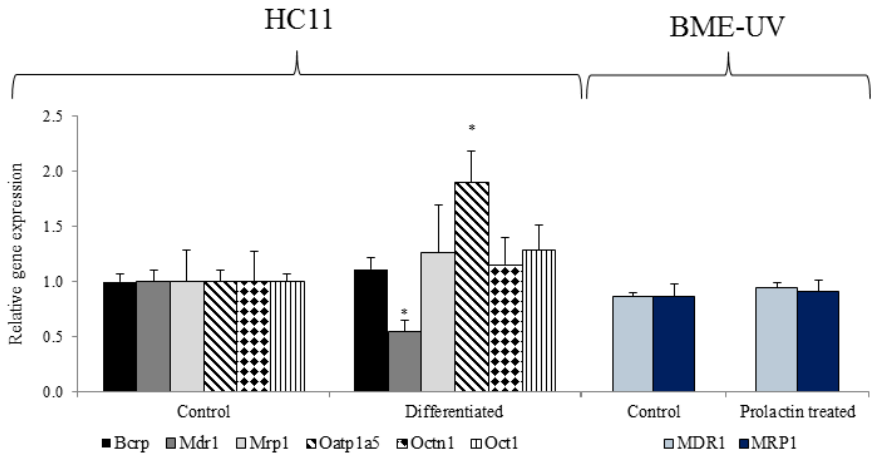

Figure 3. Relative gene expressions of transporters in undifferentiated (control) and differentiated HC11 cells (left) and prolactin treated BME-UV cells (right). Statistically significant differences as compared to controls *p ≤0.05.

In addition to gene expressions, Bcrp- and Mdr1-protein was detected in both undifferentiated and differentiated HC11 cells (paper I). Bcrp protein expression was up-regulated and Mdr1 protein expression down-regulated in the differentiated as compared to the undifferentiated HC11 cells (paper I). These results are in line with the in vivo findings that BCRP is up-regulated and MDR1 down-regulated of lactating mammary glands of various species (Gilchrist & Alcorn, 2010; Jonker *et al.*, 2005; Alcorn *et al.*, 2002) and suggest that the HC11 model can be used to both assess function of endogenous Bcrp and Mdr1 to detect new substrates as well as inducers and inhibitors of these transporters.

HC11 cells have been used as a model for studies on hormonal regulation of mammary epithelial cell differentiation as well as milk protein gene expression and secretion (Kabotyanski *et al.*, 2006; Desrivieres *et al.*, 2003; Wartmann *et al.*, 1996). Furthermore, HC11 cells have been applied to examine the impact of cadmium on lactating mammary cells (Öhrvik *et al.*, 2006) and also to characterize some transporters belonging to the Solute Carrier (SLC) family and ion channels, which are implicated in the flux of magnesium, zinc, copper and calcium across the membranes of the lactating mammary epithelium (Ross *et al.*, 2013; McCormick & Kelleher, 2012; Öhrvik *et al.*, 2011; Wolf *et al.*, 2010; Boyd & Naray-Fejes-Toth, 2007; Kelleher & Lonnerdal, 2006; Kelleher & Lonnerdal, 2005). However, the HC11 cell model has not yet been used to characterize expression and/or function of ATP Binding Cassette (ABC) transporters. The localization of both BCRP and MDR1 in the apical membranes of mammary epithelial cells in combination with the promiscuity

of these active transporters has risen considerable concern from public health, food safety, and regulatory perspectives about the presence of drugs and toxic compounds in milk both for breast fed infants as well as for consumers of dairy products (Wassermann *et al.*, 2013).

In paper II we identified gene expressions of Mrp1, Oatp1a5 (the mouse orthologue to human and bovine OATP1A2), Octn1 and Oct1 in HC11 cells with a secreting phenotype. Conceivably, the HC11 cell model may also be applicable to investigate substrates as well as inducers and inhibitors of these other ABC and SLC transporters. In the bovine mammary epithelial BME-UV cells only MDR1 and MRP1 gene expressions were detected. The lack of expressions of BCRP, OATP1A2 and OCTN1 in the BME-UV cells may be due to a number of reasons such as loss of transcription factors and selection of cells during passages, higher passage numbers or culturing conditions (Monzani *et al.*, 2011; Blum *et al.*, 1989; Larson, 1976).

4.4 Effects of cell differentiation, inhibitor and Bcrp RNAi on BCRP-mediated transport (paper I)

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

Our results showed that the net accumulation of MX was higher in the differentiated HC11 cells as compared to the undifferentiated controls despite an apparent higher expression of Bcrp. This may, at least in part, be explained by a reduced expression of Mdr1. Although MX extrusion is predominantly mediated by Bcrp it has been demonstrated that MX can be transported to the extracellular compartment by Mdr1 although this transporter harbors less substrate specificity for MX than Bcrp and thus requires higher substrate

concentrations (Kodaira *et al.*, 2010; Rautio *et al.*, 2006; Ahmed-Belkacem *et al.*, 2005). Numerous SLC transporters, comprising both organic cation transporters (OCTs) and organic anion polypeptide transporters (OATPs), in mammary epithelial cells are affected at the transcriptional level by lactogenic stimulation (Gilchrist & Alcorn, 2010; Alcorn & McNamara, 2002) including some with potential MX affinity. It may, hence, be possible that the increased accumulation of MX in the HC11 cells featuring a secretory phenotype as opposed to the undifferentiated ones is not only connected to the reduced expression of Mdr1 observed herein, but also to a concerted action of a number of other transporters including Oct or/and Oatp transporters.

Table 2. *Accumulation and secretion of ^{3}H-mitoxantrone in undifferentiated, differentiated and BCRP siRNA transfected HC11 cells. The data are presented as pmol mitoxantrone/mg cellular protein.*

Experimental condition	Accumulation	Effect of GF120918 on Accumulation	Secretion	Effect of GF120918 on Secretion
Undifferentiated	521 ± 86[a]	-	63 ± 9[e]	-
Undifferentiated + GF120918	631 ± 45[b]	121%	41 ± 2[f]	65%
Differentiated	655 ± 125[c]	-	103 ± 7[g]	-
Differentiated + GF120918	893 ± 153[d]	136%	71 ± 10[h]	69%
Undifferentiated Mock	505 ± 30[i]	-	-	-
Undifferentiated RNAi	1023 ± 42[j]	-	-	-

[bc]Statistically significant increased accumulation as compared to **a**, $p \leq 0.05$;

[d]Statistically significant increased accumulation as compared to **c**, $p \leq 0.01$;

[f]Statistically significant decreased secretion as compared to **e**, $p \leq 0.01$;

[g]Statistically significant increased secretion as compared to **e**, $p \leq 0.01$;

[h]Statistically significant decreased secretion as compared to **g**, $p \leq 0.01$

[j]Statistically significant increased accumulation as compared to **i**, $p \leq 0.01$.

4.5 Effects of prochloraz on gene expression and function of transporters (paper II)

4.5.1 Gene expression

In the present investigation the imidazole fungicide prochloraz (N-propyl-N-[2-(2,4,6-trichlorophenoxy)ethyl]-1H-imidazole-1-carboxamide) used in horti-

and agriculture (EFSA, 2011) was chosen as a test compound to examine the effect on the transporters expressed in the HC11 and BME-UV cell lines. In differentiated HC11 cells, prochloraz treatment at non-cytotoxic concentrations resulted in a down regulation of gene expressions for Bcrp, Oatp1a5 and Octn1 (Figure 4, left). Mdr1 mRNA levels were significantly induced at the two highest concentrations of prochloraz (10 and 30µM) (Figure 4, left). No statistically significant differences were observed for gene expressions of β-casein, Mrp1 and Oct1 after prochloraz exposure (Figure 4, left). In BME-UV cells, gene expressions of MDR1 and MRP1 were induced at the highest concentration of prochloraz (30 µM) (Figure 4, right).

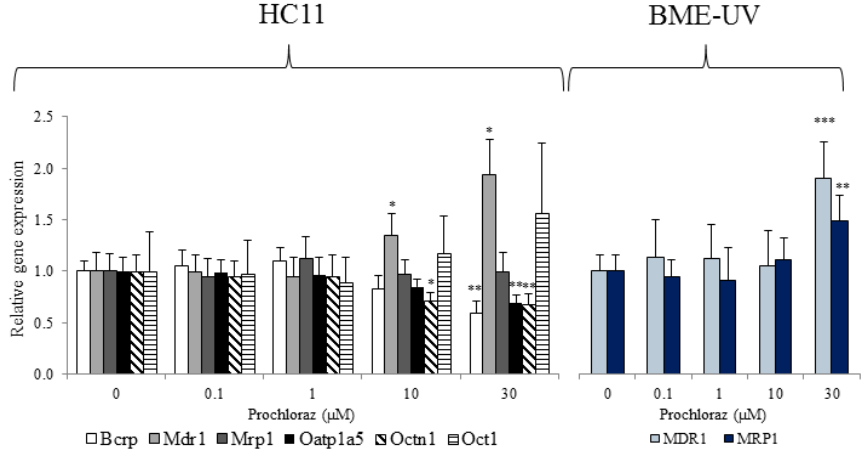

Figure 4. Relative gene expression of transporters following prochloraz treatment in differentiated HC11 and confluent BME-UV cells. Statistically significant differences as compared to controls *p ≤0.05; **p≤0.01; ***p≤0.001.

Prochloraz has been reported to increase Bcrp expression in primary bovine mammary epithelial cells (Halwachs *et al.*, 2013) and is transported to milk (Vinggaard *et al.*, 2005). However, in the differentiated HC11 cells prochloraz did not induce, but rather reduced Bcrp expression. Causes for this discrepancy can only be speculated upon but may depend on differences in experimental conditions such as prochloraz concentrations used, exposure times, or species differences in the display of transcription factors and regulation in BCRP gene expression. It can be noted that (Tan *et al.*, 2010) reported that TCDD treatment resulted in induction of human but not murine BCRP transcripts.

Interestingly, the results obtained in the present study showed that prochloraz induced gene expression of Mdr1 and MDR1 in both differentiated

HC11 and BME-UV cells, respectively. In addition, MRP1 expression was upregulated by prochloraz in BME-UV cells. Based on predominant expressions of MDR1 and MRP1 in excretory organs which have roles in elimination of drugs and xenobiotics; one possible explanation can be that both MDR1 and MRP1 undertake protective roles as efflux pumps in order to prevent accumulation of chemicals and toxic effects in the cells. The localization and induction of MDR1 in the apical membranes of mammary epithelial cells may consequently result in an active transport of toxic compounds into milk. It has been shown that digoxin is a MDR1 substrate (Taipalensuu *et al.*, 2004).

4.5.2 Function

Differentiated HC11 and confluent BME-UV cells were exposed to 30 µM prochloraz for 24h as described above and the function of MDR1 was studied using digoxin as a substrate. Accumulation of digoxin was significantly reduced in prochloraz treated HC11 cells compared to the controls cells. Also in BME-UV cells a lower, although not statistically significant, accumulation of digoxin was observed after prochloraz treatment (Figure 5).

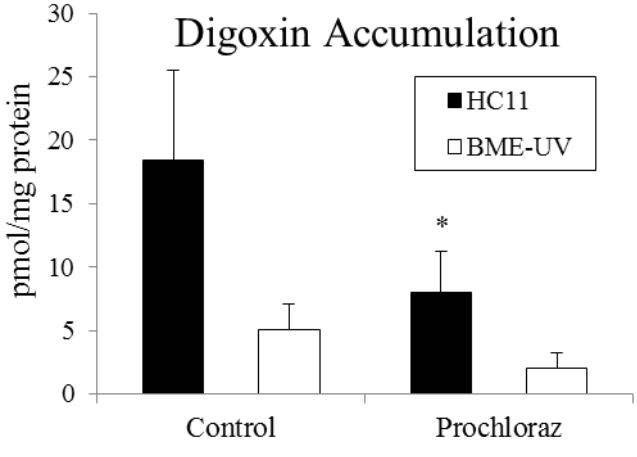

Figure 5. The accumulation of ^{3}H-digoxin in differentiated HC11 and confluent BME-UV cells, treated with 30 uM prochloraz for 24h as described in Paper II. Statistically significant differences as compared to controls *p ≤0.05.

The increased MDR1 expression in prochloraz exposed mammary epithelial cells demonstrates that chemicals may disrupt the expression and function of transporters and increase secretion of hazardous chemicals in milk.

5 Conclusions

Conclusions from the combined results from papers I and II are:

- Gene expressions of ABC- and SLC- transporters differ at various stages of mammary gland development and probably reflect the physiological role of these proteins in the delivery of essential nutrients into milk.

- Murine mammary epithelial HC11 cells feature endogenous expressions of Bcrp, Mdr1, Mrp1, Oatp1a5, Octn1 and Oct1. Gene expressions of Bcrp, Mdr1 and Oatp1a5 in undifferentiated and differentiated murine mammary epithelial HC11 cells correlate to expressions in the mammary glands of non-lactating and lactating mice. Bovine mammary epithelial BME-UV cells feature endogenous expressions of MDR1 and MRP1.

- The imidazole fungicide prochloraz causes an increased secretion of the MDR1 substrate digoxin in both differentiated HC11 and in BME-UV cells, which can be explained by induced gene expression MDR1.

- The HC11 and BME-UV mammary cell models can be used both to examine if chemical compounds are actively transported across the mammary epithelium and if they disrupt the normal function of transporters.

- The models are valuable tools for identifying substrates, inhibitors and inducers of ABC- and SLC- transporters expressed in the mammary epithelium during lactation.

6 Future Perspectives

- In the project we have developed mammary cell models for screening substrates, inhibitors and inducers of various ABC- and SLC- transporters expressed in the mammary epithelium during lactation. These models could be applied in future studies to examine if drugs and other chemicals are substrates for any of the ABC- and SLC-transporters which would result in high concentrations in milk. In addition, chemical compounds affecting expression or function of ABC- and SLC-transporters in the lactating mammary tissue may cause changes in the composition of nutrients in milk with potential adverse health effects in breastfed infants or dairy consumers.

- The present studies were mainly focused on gene expression of transporters. Studies on the protein expressions of transporters in the mammary cell models are needed.

- Little is known about localization of the ABC- and SLC-transporters in the mammary tissue. To understand the physiological role of transporters in the membranes of mammary epithelial cells during lactation studies should be performed to localize these transporters in mammary tissue.

- Mastitis and especially subclinical mastitis is a common disease in cows caused by bacterial infection. It has been shown that inflammation of tissues due to bacterial infection affect expression and function of transporters in various cell types. Thus, it would be of interest to apply the mammary cell models in infection studies to assess the impact of bacterial infection on expression and function of transporters.

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