
# Development of a cell model for studies of the secretion of xenobiotics into milk

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**Abstract**

Milk is produced in the mammary gland in small structures called alveoli. The lumen in the center of these alveoli is surrounded by alveolar epithelial cells and milk production takes place in these cells. HC11 is a murine mammary epithelial cell line deriving from non-tumorigenic mammary epithelial cells that showed normal morphology and function, such as casein induction, *in vitro* after several sub-cultures. The overall aim of the project is to develop an in vitro model for investigating the transport of toxic compounds into milk by using HC11 cells. In the first part of the project the optimal time for differentiation of the HC11 cells into a milk protein synthesizing phenotype was determined. In the second part transport studies were performed across the HC11 cells to examine the function of breast cancer resistance protein (BCRP). The optimal time for maximum differentiation was determined by measuring the expression of the milk proteins, whey acidic protein (WAP) and β-casein, through Real-Time PCR , after having cultured HC11 cells in differentiating medium. Immunohistochemistry was also performed to examine the morphology and localization of β-casein in the differentiated cells. The transport study was performed in transwells using the BCRP substrate mitoxantrone, measuring both transport across, as well as uptake into the HC11 cells. The results from quantifying the expression of WAP and β-casein, indicate that the optimal time for maximum differentiation of the HC11 cells is 72h. Immunohistochemistry also showed that at this differentiation period the HC11 cells start to form structures resembling alveoli. β-casein expression was predominantly detected in the differentiated HC11 cells closest to the alveolar lumen. The transport experiments gave no useful results concerning mitoxantrone transport across the cells but the results obtained in the uptake experiment indicate that BCRP is upregulated and active in differentiated HC11 cells. In conclusion, the results obtained in this project demonstrate that HC11 cells is not suitable for studies of transport of toxic compounds across the alveolar epithelium but seems to be a promising model for functional studies of transport proteins such as BCRP.

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## 1. Introduction

### *1.1 Mammary gland*

Typical for mammals is their ability to sustain their newborn on milk which is a complex mixture containing all required nutrients for normal development of the offspring. Milk is produced in the lactating mammary gland, in small histological structures called alveoli. Each alveolus consists of a single layer of polarized alveolar epithelial cells surrounding an alveolar lumen where the produced milk is kept (McManaman & Neville, 2003; http://classes.aces.uiuc.edu/AnSci308/Mamstructure/histology_5.html). Adjacent to the alveolar epithelial cells there is a layer of myoepithlial cells whose function is to help secreting the milk in the alveolar lumen into the lactiferous duct by contracting as a response to the release of the hormone oxytocin from the posterior pituitary gland (http://classes.aces.uiuc.edu/AnSci308/Lactation/overview.html). External to the myoepithelial cell layer there is a basement membrane made of connective tissue. Between the milk producing alveoli there is a stroma which contains for example, blood vessels, fibroblasts and leukocytes. The alveoli, which develops during pregnancy are grouped together in lobulus and surrounded by connective tissue. The lobules in their turn are clustered into lobes (http://classes.aces.uiuc.edu/AnSci308/Mamstructure/histology_2.html). The parenchymal tissue is a collective name for alveoli, ducts and connected stroma. In non-lactating mammary tissue there is, outside of the parenchyma, extensive adiopose tissue in the form of fat pads. In lactating mammary tissue this adipose tissue is almost completely absent. Most of the development of the mammary gland takes place directly after birth and after that, there are two more prominent phases. The first phase takes place at the onset of puberty when the lactiferous ducts begin to grow and the second one occurs during pregnancy and leads to alveolar differentiation (Richert et al, 2000).

*1.2 Proliferation and differentiation of alveolar epithelial cells*

In general a cell needs a differentiating program and environmental input to transform from a undifferentiated precursor to a differentiated cell. When organ development takes place, a stem cell produces two daughter cells, of which one remains a stem cell and the other differentiates. The process in which a cell becomes fully differentiated can take place in several steps with rounds of proliferation along the way (Robinson et al, 1995).

Among the epithelial cells of the lactiferious ducts there is a type of mammary stem cells. It has been demonstrated in mice that when the ducts start to grow during puberty, they form terminal end buds (TEB). The TEBs consist of an epithelium and an outer layer of undifferentiated pluripotent stem cells, and are the site for elongation and branching of the ducts. The outer layer of these TEBs consists of undifferentiated pluripotent stem cells, called cap cells (Richert al, 2000). The TEBs reach into the mammary fat pads where growth at the tip of the buds is stimulated by estrogen (http://classes.aces.uiuc.edu/AnSci308/Mamdevelop/postpubertal.html). In humans a similar structure called, the terminal ductile lobular unit (TDLU) perform this type of development (http://classes.aces.uiuc.edu/AnSci308/Mamdevelop/postpubertal.html).

During pregnancy the hormones estrogen and progesterone causes the epithelial cells of the duct to proliferate ten to twenty times and they also causes development of the alveoli (Alberts et al, 2002). The differentiation and proliferation is determined by three different factors: hormonal signalling, cell to cell, and cell to matrix interactions. (Desrivières et al, 2003). An important event in the differentiation process is the activation of the epidermal growth factor (EGF) receptors (Desrivières et al, 2003). It has been shown that activation of the EGF receptor in HC11 cells ( a cell line of murine mammary epithelial cells, see below) is essential for preparing, and making these cells competent for the hormonal signals that induce differentiation, while at the same time inhibiting the actual differentiation process (Desrivières et al, 2003). When EGF, a polypeptide growth factor and a mitogen, promoting cell proliferation, is removed, and the activation of the receptors subsides, the differentiation

of the HC11 cells can take place after adding insulin, glucocorticoids and prolactin (Desrivières et al, 2003). Investigating mammary cell differentiation, HC11 cells were used and after being allowed to differentiate, 6o proteins showed altered protein expression levels between the two states, proliferating and differentiated. The proteins that showed the greatest difference were cytoskeletal components, like the tubulin alpha chain which is upregulated in differentiated cells; chaperons, like various members of the HSP70 (heat shock proteins) families that were usually expressed in proliferating cells; proteins involved in folding and stability like disulfide isomerase which was expressed in higher levels in proliferating cells; calcium-binding proteins like annexin II which is highly expressed in differentiated cell; proteins involved in the RNA processing pathways, like the transcription factor PCNA, which is expressed in proliferating cells and regulators of cellular metabolism like proteins involved in the glycolytic pathway (e.g. aldolase A) which were highly induced in differentiated cells (Desrivières et al, 2003).

Prolactin is a peptide hormone produced in the pituitary gland, with several different functions and it affects the cell through the trans-membrane prolactin receptor (PRL-R) which has been found in seven different isoforms, of various sizes in murine tissue (Buck et al, 1992). Of these, the biggest ones are predominantly expressed in the alveolar epithelial cells of the mammary gland and seem to be responsible for inducing synthesis of the milk proteins β-casein and whey acidic protein (WAP) (Buck et al, 1992; Triplett et al, 2005), often used as markers of differentiation. The prolactin receptor belongs to the superfamily of tyrosin-kinase-linked receptors. The receptor is functioning through the assistance of the Janus kinase 2 (JAK2), and signal transducers and activators of transcription 5 (STAT5), molecules (Gilbert, 2000), of which STAT5 is an nuclear transcription factor which induces the transcription of β-casein and WAP (Groner, 2002; Simpson & Nicholas, 2002).

Glucocorticoids are steroid hormones that belong to the corticosteroids and are produced from cholesterol in the adrenal gland. The glucocorticoids receptor (GR) is located in the cytosol since the glucocorticoids are lipophile and can enter through the membrane. In its inactive form the receptor is bound to an inhibiting protein, heat shock protein 90 (HSP90).

(Groner, 2002). When GR is activated by the hormone, it dimerizes and the dimer relocates into the nucleus (Groner, 2002). In the nucleus the activated STAT5 and GR has been believed to form a complex that binds to the STAT5 response element and enhances induction of the milk protein β-casein, compared to the induction when STAT5 alone is responsible (Groner, 2002). Recent findings suggests though, that GR is not essential for production of milk proteins *in vivo* and that glucocorticoids are involved in some alternative pathway as well (Wintermantel et al, 2005).

The peptide hormons insulin and EGF are both growth factors that are unable to pass through the membrane so they both use transmembrane receptors and receptor tyrosine kinases for signalling into the cells. (Berg, Stryer & Tymoczko, 2002).

### *1.3 Lactation*

Lactation is conducted through a cycle consisting of four different stages: mammogenesis, lactogenesis, galactopoesis and involution. Mammary growth, mammogenesis, is initiated by the onset of puberty, when the system of ducts expands rapidly. Lobules of alveoli can also grow depending on species. Further development is dependent on hormonal control, governed by the various stages of the reproduction cycle (Lamote et al, 2003). During gestation proliferation of the alveolar epithelial cells are induced by 17β-estradiol (E2) and progesterone (P) (Clarke, 2000). Lactogenesis, the onset of milk production, occurs in the first few days after parturition when withdrawal of progesterone in the presence of prolactin stimulates the alveolar epithelial cells to start producing milk (Neville, McFadden & Forsyth, 2002). Oxytocin is also involved in the process, causing the myoepithelial cells to contract and thus emptying the contents of the alveolar lumen (Neville, McFadden & Forsyth, 2002). The maintenance period, galactopoeisis, occurs when suckling is stimulating the milk production and the main hormones controlling this is prolactin and somatropin. Both of these hormones are important for lactation but which one that dominates is depending on species. Among rodents and humans prolactin is most important and among ruminants it is

somatropin (Lamote et al, 2003). Involution, i.e. the regression of the mammary gland, is initiated by withdrawal of suckling, or milking, which leads to a decrease in the secretion of the hormones that maintain milk production. Subsequently the feedback inhibitor factor (FIL), inhibits the synthesis of milk protein such as casein and lactose through an autocrine feedback mechanism. (Lamote et al, 2003; Blatchford, Hendry & Wilde, 1998) The first step of involution is completed with the onset of apoptosis of the alveolar epithelial cells. In the second and irreversible step of involution the apoptotic activity is increased through an increased expression of metalloproteinases leading to proteolysis of the extra cellular matrix and remodelling the mammary gland to its pre-pregnancy resting state (Streuli & Gilmore, 1999).

### *1.4 Milk proteins*

There are two major groups of the milk proteins produced by the mammary epithelial cells: the specific milk proteins caseins and the more general whey proteins, which is the collective name for all proteins left in the supernatant when caseins have been pelleted after ultra centrifugation of skim milk. Whey proteins can be specific milk proteins like whey acidic protein, but they can also be more general proteins like, immunoglobulins for example. Which one of the two groups that is predominant varies with species. In humans whey proteins are most common and in mice the caseins dominate (Bouguyon et al 2006). The general function that milk proteins have in common, is to provide the offspring with amino acids. Additional functions of the caseins are to provide offspring with phosphate and calcium (Rasmussen et al 1999). The caseins forms micelles with a hydrophobic core in which they can store and transport phosphate and calcium. The additional functions of the whey proteins are more complex and diverse, and not always fully understood. Among the milk proteins, two are of particular interest for this study; β-casein and the whey protein WAP.

Low levels of milk proteins are produced in undifferentiated cells but, the major production is initiated by pregnancy. In the mouse β-casein is relatively early, making its appearance day 10-12 of pregnancy, whereas WAP is somewhat later, about day 14-15 (Simpson & Nicholas, 2002). Both expression of WAP and β-casein are believed to be basically regulated by insulin, glucocorticoid and prolactin (Vonderhaar & Ziska, 1989).

Of the three hormones involved in the induction of β-casein expression; insulin, prolactin and glucocorticoids, insulin is the only one that has no effect on its own. Both prolactin and glucocorticotids induce expression on their own in vitro, but the effect increases when they work together (Doppler, Groner & Ball, 1989). Insulin enhances the expression together with prolactin but not with glucocorticoids, (Doppler, Groner & Ball, 1989) but apparently the expression of β-casein increase, if insulin is present when the induction, performed by prolactin and glucocorticoids take place (Groner & Gouilleux, 1995).

All functions of WAP are not entirely understood but one of them at least, seems to be to inhibit proliferation in mammary epithelial cells by inhibiting cyclin D1 which is important for moving the cell from the G0/G1 phase to the S1 phase of the cell cycle. (Nukumi et al, 2004). WAP is expressed exclusively in the latter part of pregnancy and in the lactating mammary gland and have therefore been used as an advanced differentiation marker (Triplett et al, 2005; Robinson et al, 1995), because halted proliferation is typical for differentatied mammary epithelial cells. The WAP proteins contain several cystein rich regions called four-disulfide core (4-DSC) domains. This domain is also present in a number of proteins with protease inhibiting function (Simpson & Nicholas, 2002). Therefore it has been suggested that WAP also could function as a protease inhibitor but so far it has not been shown. Pups of mice with the WAP gene knocked out has been showed to grow poorly during the second half of lactation so the protein is of importance to the offspring as well (Triplett et al, 2005).

In culture of mammary tissue WAP requires insulin, cortisol and prolactin for expression, just like β-casein. Prolactin is the main inducer but WAP seems to be regulated

independently of β-casein, mainly using a different member of the STAT family, Stat5a whereas β-casein is highly expressed using Stat5b (Simpson & Nicholas, 2002).

Prolactin and glucocorticoids work together with a synergetic effect in inducing the production of β-casein in cell and tissue culture (Doppler, Groner & Ball, 1989). In vivo though, it has been shown that mice lacking the glucocorticoid receptor (GR) in epithelial cells were able to produce the necessary amount of both WAP and β-casein for their pups. What was affected by this GR deficiency was mainly the lobuloalveolar development (Wintermantel et al 2005).

There are other signal pathways involved as well, which have been demonstrated to be involved in regulation of the milk protein production such as the one governed by transcription factors C/EBPs, NFI, YYI and others (Groner, 2002).

### *1.5 Transport over the mammary epithelium*

There are five different pathways for transport over the mammary epithelium and these are the same pathways that are used for secretion of proteins, lipids, ions, nutrients and water into milk. The exocytotic pathway is the main route for milk protein, oligosaccharides and various nutrients like lactose, phosphate and calcium packaged in secretory vesicles in the Golgi and then released from the apical side of the cell. This pathway is for example used by caseins that are released as miscelles into the lumen and it is quite similar to exocytotic pathway of other cells (McManaman & Neville, 2003). Milk lipids are produced in the smooth endoplasmatic reticulum as protein coated micro lipid droplets (MLD). MLD are secreted coated in a bi-layer membrane, derived from the cell itself, as milk fat globules (MFGs) in a budding mechanism unique for mammary epithelial cells (McManaman & Neville, 2003; Mather & Keenan, 1998). The transcytotic pathway is a way to transport various macromolecules such as immunoglobulins through the cell. The molecules are transported as endosomes and released through exocytosis. The membrane transport

pathways consist of a variety of transport mechanisms for ions and small molecules such as glucose and amino acids (McManaman & Neville, 2003). These transport systems requires specific transporters at the membrane both on the apical and basal side. Ions known to use this pathway are sodium, potassium and chloride (Shennan & Peaker, 2000). The paracellular transport pathway transports various molecules of different sizes between the alveolar lumen and the interstitial space via leaky tight junctions during pregnancy. Due to tightened tight-junctions this pathway is closed during lactation, triggered by progesterone withdrawal in the presence of some glucocorticoid and prolactin (Nguyen, Parlow & Neville, 2001).

The tight-junctions between the epithelial cells functions as a barrier during lactation, but there is still a risk that drugs or toxic compounds could find their way into milk through the transport pathways that goes through the cells, and lipohilic compounds can enter milk through transcytosis.

### *1.6 BCRP and PGP*

The breast cancer resistance protein (BCRP) and p-glycoprotein (PGP) are ATP-binding cassette (ABC) proteins. The ABC super family consists of transmembrane proteins involved in transporting a large number of substrates across cell membranes. Some of these proteins, among them BCRP and PGP, are drug efflux proteins, who prevents high intracellular concentrations of substrates by pumping them out (Staud & Pavek, 2005). Among them are some that have been connected to multidrug resistance, and in both human and mice these are specifically, PGP (the gene is called MDR1), multidrug resistance associated protein 1 (MRP1) and BCRP (Staud & Pavek, 2005). Unlike MDR1 or MRP1, which are down regulated in the lactating mammary tissue, BCRP are induced during pregnancy and especially lactation on the apical side of the mammary epithelium which makes it a major suspect for the transport of drugs or toxins into milk. (Jonker et al, 2005). In spite of its name BCRP are located not only in breast cancer cells, but also in stem cells, other cancer cells and epithelium in various parts of the body (Staud & Pavek, 2005) PGP mainly

transports small hydrophic molecules after they have diffused into the cell through the cell membrane. It is located in most parts of the body but is specifically abundant in the liver, intestines and the kidney.

The drug efflux proteins tend to overlap considerably both when it comes to localization and substrate specificity, as well as known inhibitors (Staud & Pavek, 2005; Taipalensuu et al, 2004). There is a variety of known substrates for BCRP, for example aflatoxin B1, ocratoxin A, acyclovir,cimetidine, PhIP and anti tumour drugs like mitoxantrone, (Staud & Pavek, 2005: Jonker et al, 2005).

### *1.7 HC11 cells*

The HC11 cell line was derived from the COMMA-1D mouse mammary epithelial cell line (Ball et al 1988), which in turn was derived from mammary tissue of BALB/c mice in the middle of pregnancy. The COMMA-1D cell line was the first non-tumorigenic line of mammary epithelial cell lines that showed normal morphology and function, such as duct morphognesis after transplantation into mammary fat pads of syngeneic mice and casein induction, *in vitro* after prolactin treatment and several sub-cultures (Danielson et al 1984). The HC11 cell line was developed to study prolactin activity at a molecular level. A characteristic that was early described for this cell line was that unlike previous ones it does not need added complex extra cellular matrix or co-cultivation with fibroblasts or adipocytes to be able to produce β-casein after prolactin treatment (Ball et al 1988). Confluence is necessary for HC11 cells to respond to the lactogenic hormones and start to differentiate (Doppler, Groner & Ball, 1989).

Apart from studies concerning their normal function as mammary epithelial cells, HC11 has also been used for various studies on breast cancer. There have, for example, been done studies on how antagonists of the different estrogen receptors affect proliferation and

apoptosis in HC11 cells (Helguero et al, 2005) as well as a study on how hypoxia, a common result of tumours, induces cyclin D1 which induces proliferation (Joung et al, 2005).

## 2. Aims of the study

The overall aim of the project is to develop an in vitro model for investigating the transport of toxic compounds into milk by using a murine mammary epithelial cell line , HC11.

In the present study the first aim was to determine the optimal time for maximum differentiation of the HC11 cells into milk protein synthesising and secreting phenotype. To assess this stage of differentiation, quantitative gene expression of two different milk proteins, β-casein and whey acidic protein was measured.

B-casein immunohistochemistry on maximally differentiated HC11 cells was performed to detect this protein and also to visualize and more closely examine the morphology of the differentiated cells.

The second aim was to perform transport studies, using the BCRP substrate mitoxantrone on fully differentiated HC11 cells, investigating the BCRP function of HC11 cells, since *in vivo* studies on BCRP expression have shown that BCRP protein is upregulated during lactation (Jonker et al, 2005).

## 3. Material and Methods

### *3.1 Cell Culture*

HC11 mouse mammary cells, passage 26, was a generous gift from Dr Shannon L. Kelleher, Department of Nutrition, University of Carlifornia, Davis and used by permission of Dr

Bernd Groner, Institute for Biomedical Research, Frankfurt. The cells were grown in growth medium consisting of: RPMI 1640 + L-Glutamine, + 25 mM HEPES, (Gibco, Invitrogen, Carlsbad, USA), $NaHCO_3$ 8g/l, gentamicin 12,5 mg/l (Gibco, Invitrogen, Carlsbad USA), 7.5 % sodium bicarbonate 29,3 ml/L, bovine insulin (Sigma-Aldrich, St Louis, USA) 5 mg/l EGF 10 µg/l and 10% heat-inactivated foetal bovine serum (FBS) (Gibco, Invitrogen, Carlsbad USA) at a pH between 7.3 and 7.4. The culture was grown at 37°C in 5% $CO_2$. Medium was changed every 2-3 days. (Monday, Wednesday, Friday).

When cells had reached 80-90 % confluence, they were passaged in the following way: All medium was withdrawn and the cells were washed in 1x PBS, 5 -7 ml/ 25 $cm^2$. PBS was removed and Cell Dissociation Solution (Sigma-Aldrich, St Louis, USA) was added 1 ml per 25 $cm^2$. The cells were incubated in 37°C until cells dispatched. After that, medium was added and the solution was suspended by pipetting up and down. The solution was centrifuged at 450 G (1500 rpm) using a GS-GR Centrifuge (Beckman Coulter, Fullerton, USA) for 5 min at 4°C. The supernatant was removed carefully. The pellet was solved in medium by pipetting up and down.

*3.2 Differentiating cells*

When confluent, cells (p.30) were differentiated by exchanging growth medium for differentiating medium (growth medium – EGF, -FBS, + prolactin (Sigma-Aldrich, St Louis, USA) 1 µg/ml, +cortisol (Sigma-Aldrich, St Louis, USA) 1µM) The cells were grown for 3, 6 12, 24, 48, 72 and 96 hours in 25 $cm^2$ bottles. At each time point, two bottles of differentiated cells and two bottles of undifferentiated cells grown in regular growth medium, as controls, were collected.

*3.3 Extraction of RNA*

Medium was removed and the cells were washed with 7 ml of 1 x PBS. To one control and one differentiated bottle 3,5 ml of Trizol® Reagent (Invitrogen, Carlsbad USA) was added and the cell homogenate was immediately stored in -70°C. Prior to the RNA extraction the cell homogenates were thawed, and two samples of 830µl were taken. 200 µl of chloroform (Sigma-Aldrich, St Louis, USA) containing isoamylalcohol (24:1) were added and the samples were vortexed thoroughly and then incubated on ice for 15 min. Next the samples were centrifuged for 15 min at 15 000 x g at 4°C in an Eppendorf centrifuge 5415 R, (Eppendorf AG, Hamburg, Germany). As a consequence of the spinning, the content was divided into 3 separate phases. The water phase on the top contains the RNA and from this 300-400 µl was taken and added to new eppendorf tubes containing 1 ml isopropyl alcohol, kept on ice. The tubes were inverted a few times and then incubated at -20°C over night for precipitation of the RNA.

The next day the samples were centrifuged for 15 min at 15000 x g at 4°C in. The supernatant was removed and the pellet was washed in 1ml of 75% ethanol (in DEPC-treated water), pipetting up and down. Next the samples were centrifuged at 7500 x g for 10 min at 4°C. The supernatant was removed and the pellets were air-dried under plastic film for 5 min. Finally the pellet was resuspended in DEPC-treated water.

To control if the process was successful and the RNA had not been degraded, some samples were electrophoresed on a 1 % agarose (Sigma-Aldrich, St Louis, USA) gel for approximately 2 hours at 60V. Figure 1 below shows the typical two thick bands of intact ribosomal RNA.

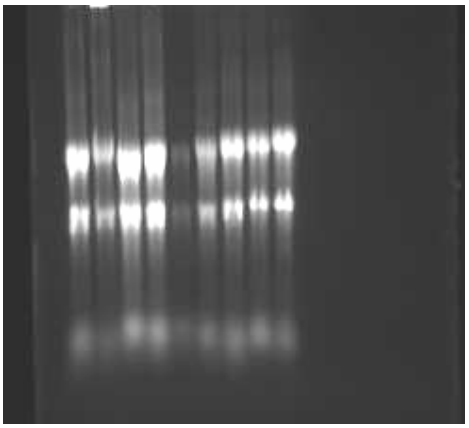

*Figure 1.* Typical bands of intact ribosomal RNA.

*3.4 RiboGreen*

RiboGreen® RNA-Specific Quantitation Kit with DNase I (R-32702), (Molecular Probes, Invitro, Eugene, USA) was used to determine concentration of the extracted RNA. Standards prepared in TE-buffer of 0, 20, 50, 100, 250, and 500 ng/ml were used to create a standard curve for fluorescence. Two samples for each time spot, one undifferentiated and one differentiated, was measured in triplets in a Victor$^2$ 1420 multilabel counter (Perkin Elmer, Wellesley, USA) at approximately 520 nm. Each sample was diluted 1000x in DEPC-treated water prior to the analysis. The samples were treated with DNase 1 20 U/ml according to the instructions of the manufacturer. Incubation times and final preparation on plate was done according to the RiboGreen® RNA-Specific Quantitation Kit protocol.

*3.5 RT-PCR*

RT-PCR was performed, using the Reverse Transcription Core Kit, (Eurogentec, Seraing, Belgium) to create cDNA from all the samples. Mix for the reactiono was: 1x Reaction buffer, 5 mM Mg, 500 µM each of dNTP, 2.5µM oligo dT, 0.4 U/µl RNAse inhibitor, 1.25

U/µl Euroscript RT, 1000 ng template RNA, DEPC water adding up to 36 µl per PCR tube. The RT-PCR program was as follows: 10 min at 25°C, 30 min at 95°C and finally 5 min at 95°C. The reaction was perfomed on an iCycler (Bio-Rad Laboratories, Hercules, USA).

*3.6 Purifying cDNA*

Purifying cDNA was done using the Strataprep® PCR Purifying Kit (Stratagene, La Jolla, USA) mainly according to the protocol with two exceptions: 600 µl DNA binding solutions was used for 100 µl PCR product and 20 µl elution buffer per microspin cup was used to elute the purified PCR procuct.

*3.7 PicoGreen*

PicoGreen was used to determine the concentration of cDNA. A master mix for 15 wells was created consisting of 1, 7 ml o TE-8 and 8,5 µl PicoGreen reagent (Molecular Probes, Invitro, Eugene, USA) according to instructions from the manufacturer. A plate was set up with triplets, adding 100 µl TE-8 for of a negative control, the standard (2,5 – 25 – 250 pg/ml) and the sample which was diluted 200 time.

*3.8 Real-Time PCR*

Real-Time PCR for the HC11 cells was run in 25 µl reactions containing 12,5 µl qPCR™ Mastermix Plus for SYBR®Green 1 – No ROX, (Eurogentec, Seraing, Belgium) 1,5 µl forward primer 5µM, 1,5 µl reversed primer 5µM, 3 µl template and 6,5 µl water. The program was: 2 min 50°C, 10 min 95°C, cycling; 40 x (60s 94°C, 60s 55°C, 45s 68°C) 7 min 68°C and final melt curve analysis 55°C to 99°C and the instrument used was a Rotor-Gene, RG-3000 (Corbett life science, Sydney, Australia). All samples, standards as well as the no template control were run in triplicates. Samples that showed melting points that did not

match the standards, as well as samples that had a higher threshold cycle value than the no-template controls.

*3.9 One-step RT-PCR*

One-step RT-PCR is a technique in which the creation of cDNA from RNA and a Real-Time PCR for quantification is perfomed in one reaction. It is a more sensitive technique for creating cDNA since the primers from the gene of interest is used instead of random oligo dT. The One-step RT PCR reaction was done using the Quanti Tect SYBR Green RT-PCR Kit from Qiagen according to the manufacturers intentions, except that: when cDNA from the standards was used, no QuantiTect RT Mix was added. 500 ng template RNA was used per 50 µl reaction. The program was 30 min 50°C, 15 min 95°C, cycling; 35 x (60s 94°C, 60s 55°C, 45s 68°C), 7min 68°C and final melt curve analysis from 50°C to 99°C using a Rotor-Gene, RG-3000 (Corbett life science, Sydney, Australia). All samples, standards as well as the no template control were run in triplicates. Known concentrations of cDNAs of the respective genes were used to create standards as described below. Samples that showed melting points that did not match the standards, as well as samples that had a higher threshold cycle value than the no-template controls, were disqualified.

*3.10 Primer design and preparing of cDNA standards*

The murine cDNAs for were obtained from the Ensemble Database (www.ensemble.org). Intron-spanning primers were designed to generate 150-250 bp PCR-products, using the Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) primer design tool, see table 1 below.

*Table 1.* Primers used in this project

| Protein | Sequences |
|---|---|
| WAP | 5' - TATCATCTGCCAAACCAACG - 3' (forward) |
| | 5' - GTCGCTGGAGCATTCTATC – 3' (reverse) |
| B-casein | 5' – CTTAACCCCACCGTCCAAT – 3' (forward) |
| | 5' – AGCATGATCCAAAGGTGAAAA – 3' (reverse) |
| BCRP | 5' – CGCAGAAGGAGATGTGTT – 3' (forward) |
| | 5' – TTGGATCTTTCCTTGCTGCT – 3' (reverse) |
| PGP | 5' – GCTGTTAAGGAAGCCAATGC – 3' (forward) |
| | 5' – AGCAATGGCGATTCTCTGTT – 3' (reverse) |

The primers were generated by CyberGene AB (Huddinge, Sweden).

Primers were diluted to the concentration of 5µM. A Real Time PCR reactions were set up as described above. As template, cDNA was used prepared from murine mammary tissue derived on lactation day 14. The products were purified using the Strataprep® PCR Purifying Kit (Stratagene, La Jolla, USA) as described above. PicoGreen was used to determine the concentration of the PCR-product. A dilution series was set up to create a standard for further determination of gene expression levels.

To control the specificity of the primers, the PCR-products were sequenced using an ABI Prism® 3100 Genetic Analyzer, (Applied bio systems, Foster City, USA). Two sequencing reaction, one for each primer, for 20 µl was set up as follows, using the Big Dye Terminator v3.1 Cycle Sequencing Kit, (Applied bio systems, Foster City, USA): 2 µl BDT v3.1 sequencer, 3 µl Supplemented 5x buffer, 5 µl Primer (1µM), 1 µl PCR-Product (3ng/µl) and 9 µl $dH_2O$. The program for the reaction was: 96°C 10 seconds, 45°C 5 seconds and 60°C for 4 minutes. To precipitate DNA, 50 µl 95% EtOH and 2 µl of 3M NaAc pH 4.6, was added to

the sequencing reaction product. The mix was vortexed and then kept in room temperature for 15 minutes, before being centrifuged for 20 minutes at 13500rpm. The pellet was washed with 250 µl 70 % EtOH and centrifuged for 5 minutes at 13500rpm. After the supernatant was removed the pellet was dried in 37°C for 10 minutes. The pellet was solved in 11 µl HiDi formamide before it was put in the ABI Prism® 3100 Genetic Analyzer (Applied bio systems, Foster City, USA). To form contigs of the sequenced product, Vector NTI®software (Invitrogen, Carlsbad, USA) was used and the forward and reverse contigs were edited and compared to known sequences of the genes using the Basic Local Alignment Sequence Tool (BLAST).

*3.11 Transport and uptake studies of mitoxantrone*

Approximately 1 million cells in 0,5 ml growth medium, cultivated in the same way as described above, were seeded on the apical side (A) of Transwell® Permeable Supports, poresize 0,4 µm and Ø 12 mm, (Corning Inc, Acton, USA) and 1,5 ml growth medium was put on the basolateral side (B) compartment of the wells. The cells were grown for seven days when the growth medium was removed and replaced with differentiation medium, as described above. The cells were allowed to differentiate for 72 hours before the transport and uptake experiments started. To examine the function of BCRP, mitoxantrone, was used as substrate. The cells were pre-incubated for 30 min at 37°C in sterile filtered Hank's Balanced Salt Solution with $CaCl_2$ and $MgCl_2$ (HBSS, Invitrogen, Carlsbad, USA) containing 25 mM N-(2-Hydroxyethyl) piperazine – N'-(2-ethanesulfonic) acid (HEPES, Sigma-Aldrich, St Louis, USA), pH 7.4, both on the A and the B sides. For the experiments with the BCRP inhibitor, the cells were pre-incubated as described above in the presence of 1µM GF120918 (Glaxo-Wellcome, Brentford, UK), a known BCRP inhibitor. 5 µM Mitoxantrone was solved in preheated HBSS solution to a final concentration of 5 µM. The plates were incubated with this solution, on both the A and B sides to prevent passive transport according to Taipalensuu et al, 2004. To examine the active transport from apical to basolateral side (AB), and the active transport from basolateral to apical side (BA). $^{3}$H-labelled Mitoxantrone, 20 000 Bq/ml, was added either on the A side or on the B side functioning as donor solution. For the

experiments involving the BCRP inhibitor, all the solutions contained 1µM of GF120918. Samples were taken from the receiver side 20, 40, 60 and 90 minutes after addition of the $^{3}$H-Mitoxantrone to the donor side. When samples were taken 600 µl was taken from the B side and replaced with pre-heated HBSS solution containing 5µM mitoxantrone at each time spot. In the same way 200 µl samples were taken from the A side in the BA experiments. At the end of the transport experiments the filtersupports with the HC11 cells were rinced thoroughly in 5 ml ice cold HBSS, removed by means of a scalpel and then placed in 1M NaOH over night for lysis of the cells. 10 ml Ultima Gold (Perkin Elmer, Wellesley, USA) was added to each sample and then vortexed thoroughly before being analyzed in a 1900 CA Tri-Carb® Liquid Scintillation Analyzer (Packard Instruments, Perkin Elmer, Wellesley, USA)

To investigate whether the cell monolayers had formed tight junctions, transepithelial electrical resistance (TEER) was measured using a Millicell® -ERS device (Millipore Corporation, Billerica, USA). TEER was also measured across a filter without cells to assess background resistance.

*3.12 BCRP and PGP gene expression in differentiated HC11 cells*

For primers see table 1. The primers were amplified through Real-Time PCR as described above and controlled by running the PCR product on a 1 % agarose gel, 60 V, for two hours. The concentration was determined through Pico-green as described above and standard concentrations were produced. One-step RT-PCR reactions were set up using RNA from HC11 cells allowed to differentate for 72 hours as described above. RNA from cells not allowed to differentate were used as controls. Both differentiated and undifferentiated cells were run in duplicate samples.

*3.13 Immunohistochemistry*

Approximately 800 000 cells in 400 µl growth cell culture medium were seeded/chamber on slides (Lab-Tec®chamber slide™system, Nalge Nunc International, Naperville, USA). In 4 of the 8 chambers on each slide the cells were allowed to differentiate for 72 hours as described above whereas the other 4 served as controls. The cells were fixed in refrigerated acetone for 5 minutes and then rinsed with PBS pH 7,4, before being incubated over night with primary antibody against β-casein (ab6408, Abcam, Cambridge, UK, diluted 1:400 in PBS). The next day the cells were washed in PBS for 3 x 3 minutes and then incubated for 30 min at room temperature with a Horseradish Peroxidase Conjugated (HRP) secondary antibody (ab6728, Abcam, Cambridge, UK, diluted 1:1000 in PBS). The slides were washed in PBS in the same way as described above and then incubated with 3.3'-Diaminobenzidine (Sigma-Aldrich, St Louis, USA) for 15 min before the staining reaction was stopped using dd$H_2O$. Finally one plate was dyed with haematoxylin. On one plate primary antibodies was omitted to check specificity. One plate was dyed with haematoxylin without being treated with antibodies for morphology studies

*3.14 Statistics*

All statistics were calculated on Microsoft Excel 2003, (Microsoft Corporation, Seattle, USA). T-tests were performed on the data comparing pg PCR product/µg RNA of the control with the differentiated sample from each timespot. P-values $\leq 0{,}05$ were considered to indicate statistical significant difference

## 4. Results

### *4.1 Determination of the optimal time for maximum differentiation of the HC11 cells*

The results showed a statistical significant upregulation of β-casein at 48 and 72 hours and downregulation at 96h (Table 2).

Table 2. β-casein gene expression in HC11 cells allowed to differentiate at various time intervals, expressed as pg/μg RNA. D = HC11 cells treated with differentiating medium. C = HC11 cell grown in growth medium.

| Time | Mean ± SD | Ratio | P-value |
|---|---|---|---|
| 3h D | 0.542 ± 0.08 | 1.49 | 0.086529 |
| 3h C | 0.363 ± 0.04 | | |
| 6h D | 0.487 ± 0.04 | 0.61 | 0.189951 |
| 6h C | 0.792 ± 0.22 | | |
| 12h D | 0.400 ± 0.07 | 0.70 | 0.240743 |
| 12h C | 0.575 ± 0.07 | | |
| 24h D | 0.500 ± 0.12 | 1.36 | 0.294687 |
| 24h C | 0.367 ± 0.09 | | |
| 48h D* | 1.475 ± 0.11 | 3.41 | 0.000777* |
| 48h C | 0.433 ± 0.66 | | |
| 72h D* | 1.508 ± 0.17 | 3.55 | 0.011571* |
| 72h C | 0.425 ± 0.00 | | |
| 96h D* | 0.233 ± 0.05 | 0.49 | 0.018499* |
| 96h C | 0.475 ± 0.00 | | |

* significant difference in gene expression between differentiated and undifferentiated HC11 cells

From literature we had learned that previous studies of differentiating HC11 cells had allowed the cells to differentiate for 48h (Kelleher & Lönnerdal, 2005). Considering this, and the results from the primary screening shown in table 2, a new test was set up, letting the HC11 cells differentiate for 48 and 72 hours respectively. This time One-step Real Time PCR was used, which is a more sensitive method. With this method β-casein showed inductions at both 48h and 72h. See table 3 below.

*Table 3.* β-casein gene expression in HC11 cells allowed to differentiate at various time intervals, expressed as pg/µg RNA, using One-step Real Time PCR. D = HC11 cells treated with differentiating medium. C = HC11 cell grown in growth medium

| Time | Mean ± SD | Ratio D/C |
|---|---|---|
| 48h D | 9.377 ± 1,50 | 1.99 |
| 48h C | 4.751 ± 2.30 | |
| 72h D | 10.523 ± 0.89 | 4.26 |
| 72h C | 2.470 ± 0.39 | |

WAP gene expression was also upregulated at 72 hours and the overall mean value of RNA levels of WAP were approximately 10 times higher in differentiated HC11 cells than in undifferentiated ones. See table 4 below. At 48h no difference in WAP gene expression was observed between differentiated and undifferentiated HC11-cells (Table 4).

*Table 4.* WAP gene expression in HC11 cells allowed to differentiate at various time intervals, expressed as pg/µg RNA, using One-step Real Time PCR. D = HC11 cells treated with differentiating medium. C = HC11 cell grown in growth medium.

| Time | Mean ± SD | Ratio D/C |
|---|---|---|
| 48h D | 32,770 ± 0.00 | 0.88 |
| 48h C | 37.205 ± 0.85 | |
| 72h D | 896.490 ± 201.40 | 33.804 |
| 72h C | 26.520 ± 2.54 | |

* significant difference in gene expression between differentiated and undifferentiated HC11 cells.

Based on the quantitative β-casein and WAP gene expression results obtained in the present study 72 hours was chosen as the optimal time for maximum HC11 cells differentiation.

*4.2 Immunohistochemistry*

Morphological differences between undifferentiated and differentiated HC11 cells after 72h of growth in differentiation media were observed, as well as showing the location of the protein β-casein in the differentiated cells. See figures 2-6 below. The differentiated cells showed a characteristic pattern, beginning to develop alveolar resembling structures with lumina, whereas the undifferentiated cells grows in monolayers. See figures 2 and 3.

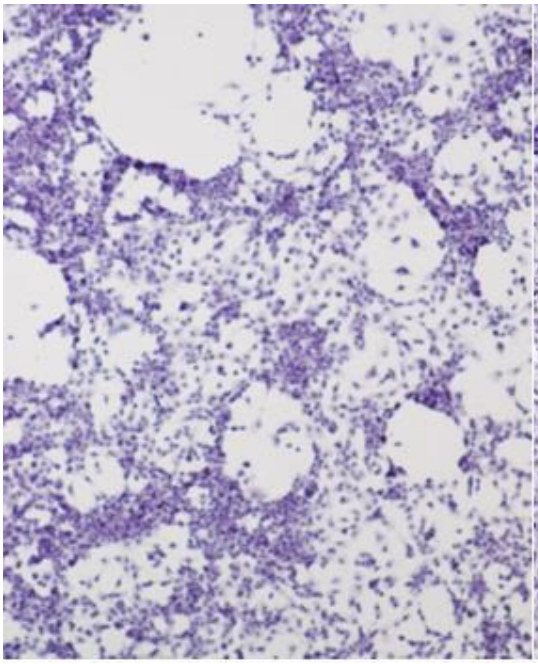

*Figure 2.* Differentiated cells (10x).

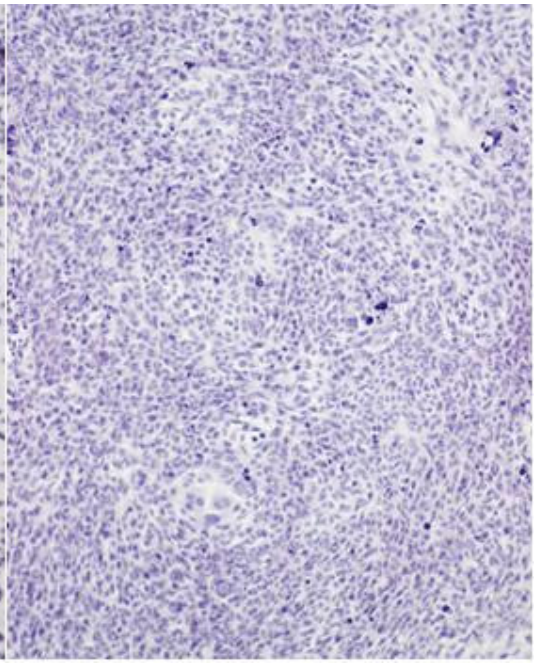

*Figure 3.* Undifferentiated cells (10x).

Haematoxylin staining showed that β-casein tended to be more expressed in the epithelia of the developing alveoli. See figures 4 and 5 below where this phenomenon is showed with two different types of staining. In both figures the darker brownish spots represents β-casein.

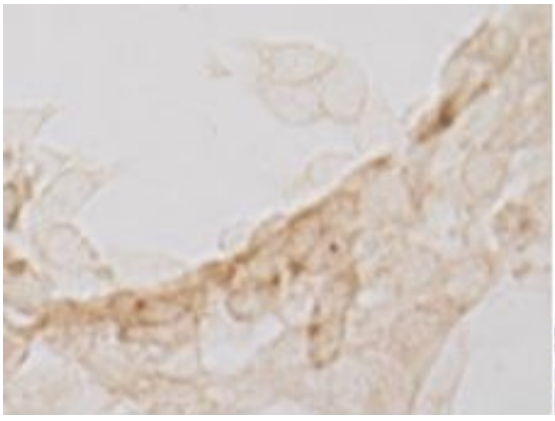

*Figure 4.* Staining with diaminobenzidine (40x).

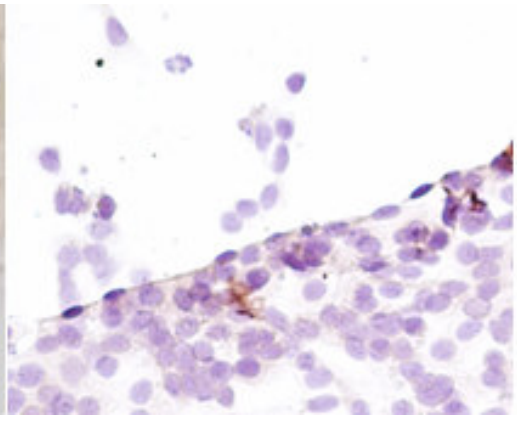

*Figure 5.* Staining with diaminobenzidine and haematoxylin (40x).

Undifferentiated cells show the presence of β-casein, but without specific location. Se figure 6 below where the light brown tone indicates a low expression of β-casein.

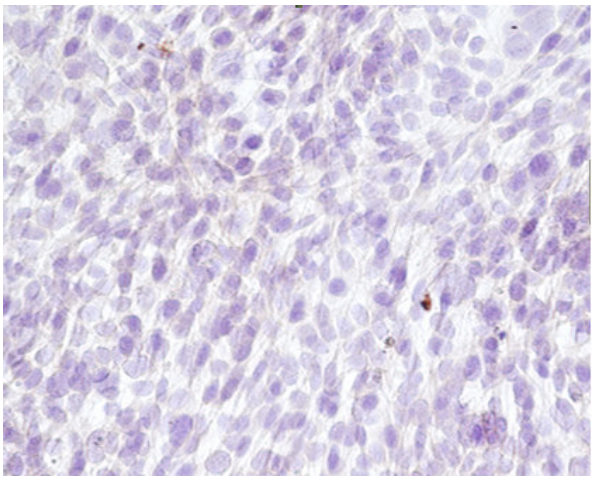

*Figure 6.* Undifferentiated cells after immunohisto and staining with both diaminobenzidine and haematoxyling (20x).

It can be noted that not diaminobenzidine staining was observed on the slide not incubated with the primary β-casein antibody.

*4.3 Drug transport*

To study drug transport and uptake in differentiated HC11 cells, mitoxantrone was used. This is a BCRP-substrate which has been shown to be upregulated during lactation *in vivo* (Jonker et al, 2005). The method used was according to Kelleher & Lönnerdal, 2005 and Taipalensuu et al, 2004. Below are the results shown of transport with or without the inhibitor GF120918. Figure 7 shows the transport from apical to basolateral side and figure 8 shows transport from basolateral to apical. The values are almost three times higher from basolateral to apical

side that from apical to basolateral. Values from all the different samples were used for a T-test, comparing the samples with or without inhibitor GF120918, where samples without inhibitor were controls. No significant differences were detected since no value was below 0,05.

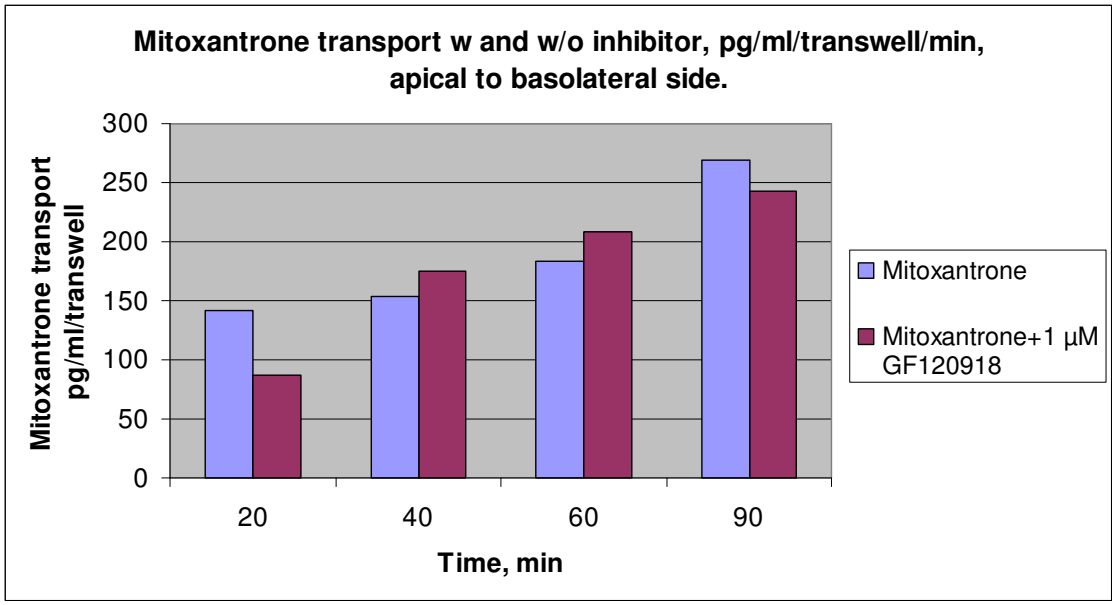


*Figure 7.* Transport from apical to basolateral side, with and without inhibitor, after 20, 40, 60 and 90 minues. No significant differences were detected.

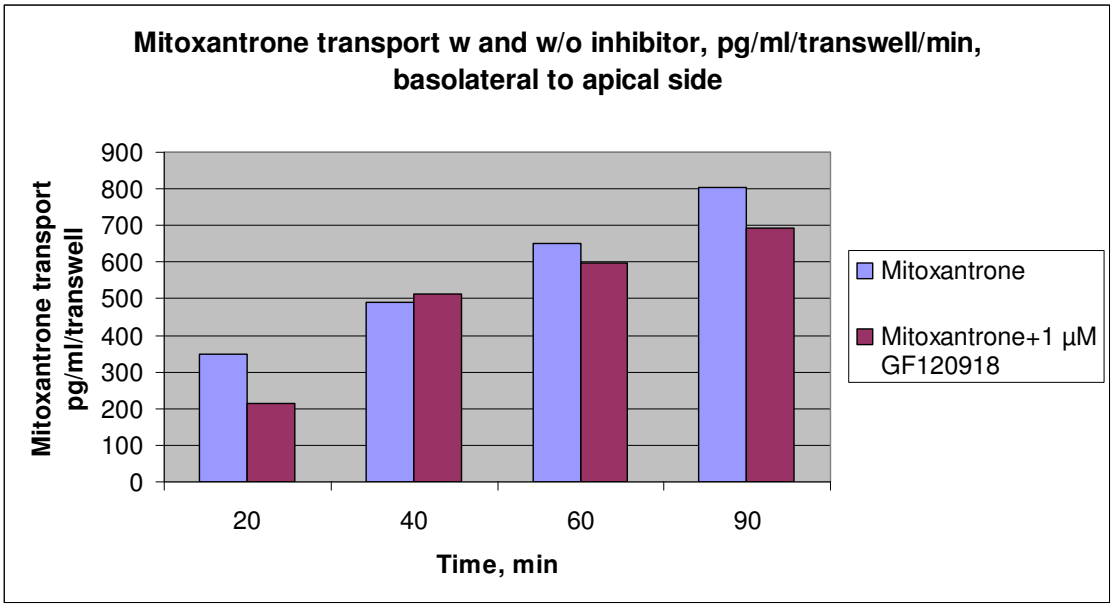


*Figure 8.* Transport from basolateral to apical side, with and without inhibitor. No significant differences were detected.

TEER over the cell layer was measured and gave the following results. See table 5. These results show a very small difference between differentiated and undifferentiated cells as well as a very small difference between cell layers and no cell control. This indicates leakiness of some sort.

*Table 5.* Average TEER-values, Ω, over the cell layer measured in Transwells containing, undifferentiated, differentiated and just medium respectively.

| Sample | Average Ω |
|---|---|
| Undifferentiated | 172 |
| Differentiated | 207 |
| Medium | 170 |

*4.4 Drug uptake*

The filters in the transwells were recovered and put in 1M NaOH to lysate the cells. The amount of radio labelled mitoxantrone was the measured in the different samples with or without inhibitor and both from apical to basolateral (A) and from basolateral to apical (B) side. A T-test was performed comparing the uptake with and without GF120918. See table 6.

*Table 6.* The uptake of mitoxantrone in pmol per filter with and without inhibitor GF120918, both from apical (A) and from basolateral (B) side.

| | pmol per filter±SD | P-value |
|---|---|---|
| Mitoxantrone A | 686 ± 164,0 | 0.0209915* |
| Mitoxantrone A + GF120918 | 1105 ± 14,6 | |
| Mitoxantrone B | 1085 ± 164,0 | 0,1057987 |
| Mitoxantrone B + GF120918 | 1313 ± 121,0 | |

*significant difference in uptake with inhibitor.

Table 6 above implies that the uptake is higher with inhibitor but the T-test values only confirms this for the apical to basolateral sample.

*4.5 Detecting BCRPand PGP through one-step PCR*

One-step Real-Time PCR was run to detect BCRP and MDR1 in HC11 cells, both differentiated and undifferentiated. Small levels of BCRP was detected but no statistically significant differences in BCRP gene expression between differentiated and undifferentiated cells could be detected. See table 7 below

For MDR1 it was possible to see a distinct difference between differentiated and undifferentiated cells where it seemed as if MDR1 was down regulated when the cells were differentiated. This effect also seemed to increase over time. Se table 7 below.

*Table 7.* Expression of BCRP and MDR1 expressed as pg/µg RNA. D = HC11 cells treated with differentiating medium for 72h. C = HC11 cell grown in growth medium.

| Time | Mean ± SD | Ratio D/C |
|---|---|---|
| *BCRP* | | |
| 72h D | 50.30 ± 8.93 | 0.974 |
| 72h C | 51.62 ± 0 | |
| *PGP/MDR1* | | |
| 72h D | 483.09 ± 46.57 | 0.493 |
| 72h C | 979.64 ± 69.35 | |

## 5. Discussion

Previous studies has generally considered HC11 cells fully differentiated after 48h in differentiating medium (Kelleher & Lönnerdal,2005), and historically have the regulation of β-casein been the major factor for determining differentiation. (Ball et al, 1988). Ball et al used a different method for quantification of gene expression. They used immunoblotting and that could be a reason for the difference between their results and this study. In this study, examining both β-casein and WAP it was demonstrated, that when the expression pattern of both milk proteins were considered, 72 hours in differentiating medium is a more likely period for maximum differentiation.

The morphological studies performed in the present study showed that HC11 cells tend to form alveolar resembling structures when treated in differentiation medium for 72h. The results also shows that β-casein protein can be detected primarlily in the differentiated HC11 cells and in those, mainly concentrated in cells closest to the lumen of these alveolar resembling structures.

The transport study with mitoxantrone A→B and B→A, with or without BCRP inhibitor GF120918 showed no difference between differentiated and undifferentiated cells, which indicates high permeability in spite of the tightened tight junctions differentiated cells normally form. (Nguyen, Parlow & Neville, 2001). Together with the very low TEER-values for the differentiated HC11 cells, this indicates a leakage over the cell epithelia. This corresponds with the morphological findings that the differentiated cells forms alveolar resmbling structures. Nevertheless, it might be possible that the HC11 cells forms very tight, tight junctions between the cells closest to the lumen of the alveolar resembling structures.

The uptake study showed a significant higher uptake from the apical side in the presence of GF120918 but not from the basolateral side. This indicates that the cells polarize upon differentation and that BCRP is located in the membrane on the apical side of the cells. When the substrate is applied on the apical side, BCRP gets in contact with it faster than if the substrate is applied on the basolateral side. From the basolateral side the lipophilic substrate gets inside the cell before getting in contact with BCRP. Another explanation for the high P-value from the basolateral side, could be that the results of the uptake from this side are more spread, and the explanation for this could be that the filter affects the uptake from this side. But overall, an effect of the inhibitor is shown, which indicates that BCRP is expressed in differentiated cells.

Previous studies have shown through western blotting, (Jonker et al, 2005), that BCRP is up regulated on protein level during lactation. However, when studying the gene expression of BCRP after allowing the cells to differentiate for 72 hours, no difference in expression could be detected in the present investigation. This indicates that BCRP is regulated at the post

transcriptional level and may explain the differences between our results and the ones obtained by Jonker et al, 2005.

MDR1 gene expression on the other hand showed a significant down regulation after allowing the cells to differentiate for 72 hours which is in line with previous finds *in vivo* that PGP are abundant in resting mammary cells and decrease during lactation (Jonker et al, 2005).

## 6. Conclusion and future work

Due to the morphology of differentiated cells, HC11 cells are not suitable for studies of transport through the cells, but the HC11 cells appears to be a promising model for functional studies of transport proteins, mediating the transport of drugs and toxic compounds into milk. Future studies on these transport proteins, notably BCRP, in the HC11 cells will tell us more about how this works. For instance through RNA silencing which will give a closer look on how BCRP functions. It would also be of interest to study how BCRP is regulated since the results of this study indicates that it is regulated on posttranslational level.

## 7. Acknowledgements

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