Aus dem Institut für Veterinär-Physiologie
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

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Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
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Freien Universität Berlin

vorgelegt von
Katharina Sophie Mießler
Tierärztin aus Güstrow

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Dekan:	Univ.-Prof. Dr. Jürgen Zentek
Erster Gutachter:	Univ.-Prof. Dr. Salah Amasheh
Zweiter Gutachter:	Univ.-Prof. Dr. Johannes Handler
Dritter Gutachter:	PD Dr. Soroush Sharbati

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List of Abbreviations

CFTR	cystic fibrosis transmembrane conductance regulator
EGTA	Ethylene Glycol Tetraacetic Acid
I_{SC}	short circuit current
GLUT-1	glucose transporter 1
MAGUK	membrane-associated guanylate kinase
MDCK	Madin-Darby canine kidney
mmHg	millimeter of mercury
mRNA	messenger ribonucleic acid
NKCC1	sodium-potassium-chloride cotransporter 1
PCR	polymerase chain reaction
PDZ	postsynaptic density protein-95 / discs large / zonula occludens-1
R_T	transepithelial electrical resistance
SV-40	simian virus 40
TAMPs	Tight Junction-Associated Marvel domain-containing proteins
V_T	transepithelial voltage
ZO	zonula occludens

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Chapter 1: Introduction

Dairy products provide a substantial contribution to human nutrition worldwide (Prentice 2014). Since over 10,000 years, mankind breeds cattle and uses its milk (Curry 2013), but the overall production of milk has increased rapidly in the past century (Capper *et al.* 2009). In today's dairy farming, milk yields of over 10 000 kg per cow and year are not unusual (Zehetmeier et al 2012). In the udder, the cycle of milk production and milk ejection causes high hydrostatic pressure changes during lactation, which the mammary epithelium must stand up to (Rasmussen and Mayntz 1998). The mammary gland tissue provides a barrier between blood and milk that determines secretion pathways and barrier properties and is responsible to prevent an uncontrolled back-leak of milk components during an accumulation of milk (Cai *et al.* 2018). Within the blood-milk barrier, sealing of the paracellular space is provided by the tight junction. These junctions are organized in a meshwork of strands and interconnect neighboring epithelial cells at the apical-most border (Staehelin 1973). In their main function, they determine the paracellular barrier as a gate-keeper for ions and nonelectrolytes, and therefore control the paracellular pathway (Mandel *et al.* 1993). Important tight junction proteins are occludin, ZO-1, and the family of claudins. Among other functions, ZO-1 is known as a scaffolding protein that provides a link to the cytoskeleton (Fanning *et al.* 1998), whereas occludin modulates strand morphology and is therefore discussed for several regulatory functions within the tight junction (Cording *et al.* 2013). To date, 27 members of the claudin family have been described in humans (Mineta *et al.* 2011), which were identified to define the barrier properties of epithelial tissues, in dependence of their specific expression pattern (Markov *et al.* 2015).

After the accumulation of milk in mice mammary glands, changes in tight junction protein composition were observed, indicating adaptive processes of the epithelial barrier to high hydrostatic pressure conditions, *in vivo* (Markov *et al.* 2012). However, the driving force of this process remained unclear, as *e.g.* milk and its compounds are also known to affect epithelial barrier properties in general, and mammary gland epithelial cells in particular (Radloff *et al.* 2017, Stelwagen *et al.* 1998). Thus, the question remained if hydrostatic pressure is able to mechanically affect mammary epithelial barrier properties and tight junction protein composition.

In epithelial barriology, the Ussing chamber represents a powerful tool for electrophysiological analyses of epithelial barrier properties, *in vitro* (Ussing and Zerahn 1951). To approach different experimental questions, the original Ussing chamber apparatus was improved and modified in several ways, including the possibility to mount cultured cells (Hug 2002, Misfeldt *et al.* 1976). Moreover, modified Ussing chamber setups were

successfully employed to analyze pressure differences in various epithelia, before (Bogdan *et al.* 2008, Voute and Ussing 1970, Wang *et al.* 2003).

To investigate the effects of hydrostatic pressure on mammary epithelial barrier properties, this thesis focused on the following three aspects:

(1) Establishment of a modified Ussing chamber technique

Functional analyses of electrophysiological barrier properties can be accomplished with the Ussing chamber technique. Dynamical analyses of barrier function challenged by variations of hydrostatic pressure could be conducted with a customized setup. Therefore, a modified Ussing chamber setup was established as part of this thesis, providing pressure application from the apical, basal, or both sides under stable conditions.

(2) Effects of negative pressure on the mammary epithelial barrier function

Negative pressure challenges mammary epithelium during milking or suckling, and milking machines use a vacuum to force milk out of the udder. Simulation of negative pressure by application of increasing hydrostatic pressure to the basal side of mammary epithelium has taken place – reported in this thesis – with focus on electrophysiological barrier properties and integrity of the tight junction.

(3) Effects of high pressure on the mammary epithelial barrier function

Accumulation of milk in the mammary gland causes hydrostatic pressure, which can *e.g.* occur during prolonged milking intervals. To maintain the blood-milk barrier, it would be beneficial if mammary barrier function could adapt to high hydrostatic pressure conditions. This thesis focused on the regulation of mammary barrier functions during bilateral pressure incubation, including changes in tight junction composition and the effects on epithelial transport mechanisms.

Chapter 2: Literature review

2.1 Pressure conditions in the mammary gland

From dairy farming, many studies are available regarding the relationship of milk yield and milking intervals in different breeds, parities and stadiums of lactation (Alex *et al.* 2015, Stelwagen *et al.* 2013). It is well known that more than one-time milking per day increases milk yield and conversely, one-time milking per day is known to decrease milk yield (Alex *et al.* 2015, Davis *et al.* 1998, Stelwagen *et al.* 2013). Nevertheless, different reasons exist to practice once daily milking and it is still not uncommon in today's dairy farming (Stelwagen *et al.* 2013). Within the udder, milk produced by the mammary alveolar epithelium is primarily stored in the alveolar tissue, with the mammary gland cistern providing additional storage capacity (Davis *et al.* 1998, Knight *et al.* 1994). Milk accumulation in the udder of dairy cows causes hydrostatic pressure in the mammary gland, which was reported in dependence of several factors like milking interval, udder quarter, lactation stage and measuring technique. Pressure can range from 2 to 5.6 kPa (Graf and Lawson 1968), and to more than 6.7 kPa (Tucker *et al.* 1961). Even values of up to 8 kPa can be found in the literature (Schmidt 1971). An example of the time-dependent development of milk secretion rate and intramammary pressure is illustrated in **Fig. 1**. Furthermore, during a calf's suckling process, peaks of more than 55 kPa were recorded in the teat cistern (Rasmussen and Mayntz 1998). On the other hand, no significant pressure changes could be detected in mice alveoli up to 20 hours after cessation of milk removal, but pressure was raised in response to oxytocin (Tolkunov and Markov 1997). After the milking process, remaining intramammary pressure values of 0.5 kPa or less were detected in cows (Graf and Lawson 1968). During mechanical milking by milking machines or suckling of a calf, negative pressure is applied to the mammary gland, resulting in -15 to -20 kPa in the teat cistern during suckling (Rasmussen and Mayntz 1998). Milking machines apply vacuum to force milk out of the udder (Enokidani *et al.* 2016). Rasmussen and Madsen recommend mean pressure levels lower than -32 kPa in the short milk tube during machine milking (Rasmussen and Madsen 2000). By comparison, average pressure values of -10 to -25 kPa were recorded in mouths of suckling calves (Rasmussen and Mayntz 1998). Moreover, milking of empty teats can cause vacuum amounts of -30 to -40 kPa in the mammary gland cistern (Rasmussen *et al.* 1994). Overall, mammary gland tissue is exposed to high hydrostatic pressure changes during the cycle of milk production and milk removal, which can reach peaks of more than 100 kPa (Rasmussen and Mayntz 1998). Despite that, mammary alveolar epithelium is able to maintain basic milk composition even during prolonged times of milk accumulation, though minor changes in composition occur, *e.g.* an increase in milk fat content after 16 hours of milk stasis (Ayadi *et al.* 2004).

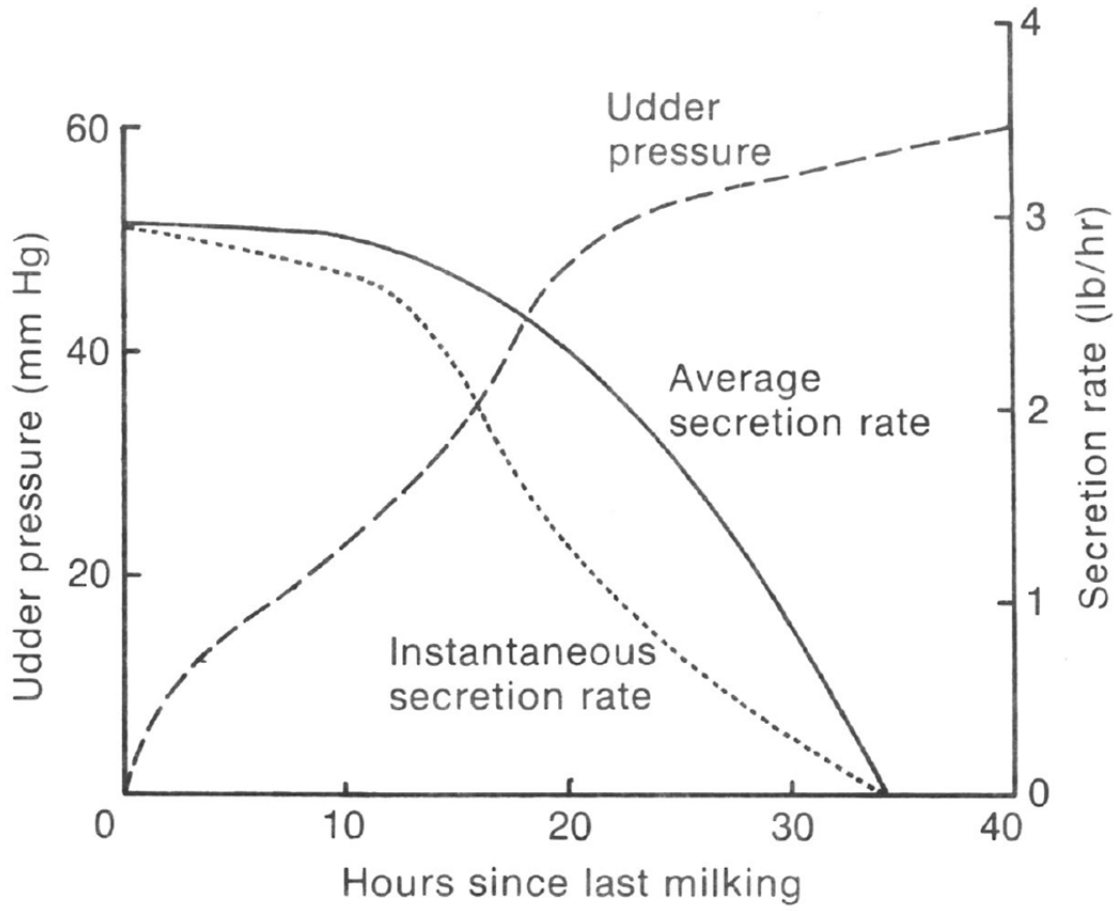

Figure 1: Correlation of milk secretion and intramammary pressure

In the first few hours after milking, intramammary pressure increases fast due to high milk secretion rate. With increasing intramammary pressure, milk secretion rate declines and reaches zero at ca. 34 hours after last the milking, when an intramammary pressure of about 60 mmHg is reached, which equals 8 kPa (Schmidt 1971).

To maintain the characteristics of milk composition and fluidity despite high hydrostatic pressure-changes, mammary gland tissue needs a well-functioning barrier, preventing an uncontrolled exchange between milk, blood and interstitial fluid. The lactating mammary gland alveolar epithelium is structured as a bi-layered epithelium, composed of a continuous cuboidal secretory cell layer and an underlaying discontinuous myoepithelial layer, which are separated by a basement membrane from the surrounding tissue (Pitelka *et al.* 1973). The tight junction is located between the epithelial cells, interconnecting them and providing a selective and adjustable paracellular barrier (Farquhar and Palade 1963). Thus, the blood-milk barrier is composed of endothelial cells – from blood and lymphatic systems –, mammary epithelial cells, and the paracellular pathway – sealed by the tight junction (Cai *et al.* 2018).

Five major secretion pathways are described for milk components to cross the blood-milk barrier (Cai *et al.* 2018, Linzell and Peaker 1971, Shennan and Peaker 2000):

1. membrane transport of *e.g.* ions, glucose and water, 2. exocytosis *via* Golgi apparatus of *e.g.* casein, lactose and citrate, 3. extrusion of milk fat globules, surrounded by membrane of the secreting cell, 4. transcytosis, *e.g.* of immunoglobulins in colostrum milk, and 5. the paracellular pathway, which relies on the permeability of the tight junction.

In general, fluid secretion into the alveoli mainly depends on the amount of lactose as a driving force for osmosis, which was demonstrated in alpha-lactalbumin deficient mice (Stinnakre *et al.* 1994). Several studies were performed to elucidate the mechanisms responsible to prevent a back-leak of milk compounds, and thereby characterizing the physiology of the mammary tight junction. For lactating cows (Stelwagen *et al.* 1997) and goats (Stelwagen *et al.* 1994), 18 and 21 hours of milk stasis were found to be time intervals, which caused the mammary tight junction to become leaky, respectively. However, mRNA analyses of genes related to milk synthesis indicated that reduced milk yields in continuously practiced once daily milking over five weeks were caused by downregulation of milk synthesis, and probably are not due to leakage of the tight junction (Ben Chedly *et al.* 2013). Nevertheless, tight junction leakage as a short-term effect of milk accumulation can be demonstrated by an increase of lactose in the blood plasma of dairy ruminants (Stelwagen *et al.* 1994, Stelwagen *et al.* 1997). In mice, milk accumulation was induced by pup removal for 20 hours, resulting in enlarged alveoli and flatted mammary epithelial cells, without indication of inflammation (Markov *et al.* 2012). As demonstrated, permeability of the paracellular pathway – controlled by the tight junction – is influenced by milk accumulation in several ways. However, the molecular composition of tight junction proteins in mammary epithelial tissue and their regulatory mechanisms in dependence of milk accumulation and hydrostatic pressure changes is not yet fully elucidated.

2.2 Cell junctions

Cellular junctions are a prerequisite for tissue formation, and thus, metazoan life. Today, four main forms of cell junctions are known, namely adherence junctions, desmosomes, gap junctions and tight junctions, as shown in **Fig. 2** (Levendoski *et al.* 2014). Concise, the existence of different cell-connecting structures – observed *via* electron microscopy – was discussed early (Farquhar and Palade 1963), followed on molecular level with the identification of desmoplakin, as part of the desmosome structure (Franke *et al.* 1981, 1982; Green *et al.* 1988). For adherence junctions, the dominating protein family was named ‘cadherins’ referring to their calcium-dependence (Yoshida-Noro *et al.* 1984), while components of the pore forming gap junctions were named ‘connexins’ because of their cell-

connecting function (Goodenough 1974). Based on electron microscopy, the strands organized in the apicolateral membrane of epithelial cells, which interconnect them and determine permeability of the paracellular pathway, were described as closely spaced adhesion particles, called tight junctions (Staehelin 1973). Both, the assumption of their existence due to physiological experiments and the observation of appropriate structures in electron microscopy, led to the first identification of a tight junction protein, namely occludin (Furuse *et al.* 1993). Following, the first member of the claudin-family was described by the same group (Furuse *et al.* 1998a). Later, tricellulin was isolated, a protein located in the space were three cells are connected (Ikenouchi *et al.* 2005). ZO-1 is – despite its name – not exclusive for the tight junction but interconnects tight junction proteins *via* the PDZ-domain binding scaffolding protein with actin filaments of the cytoskeleton (Stevenson *et al.* 1986, Tsukita *et al.* 2009). It is a member of the MAGUK family of proteins and can be found in diverse cell junctions (Anderson 1996).

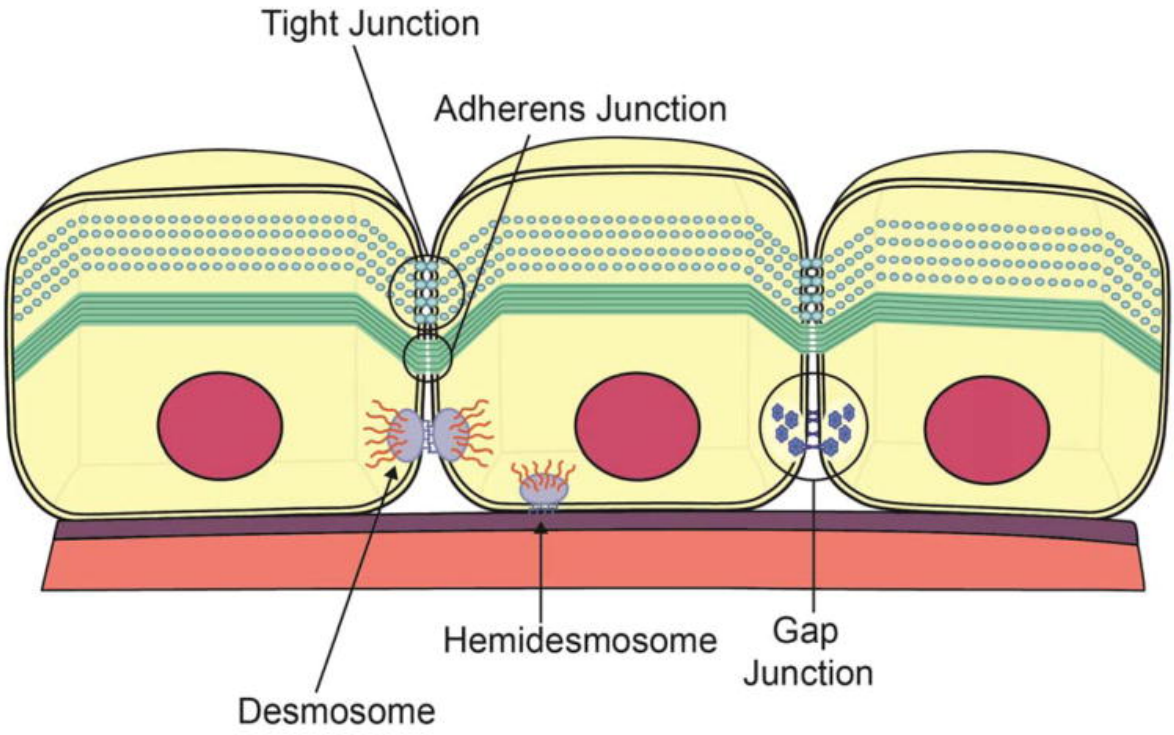

Figure 2: Epithelial cell junctions in overview

Tight junctions form the barrier determining cell to cell connections at the apical-most side of epithelial cells and are therefore classified as occluding junctions. Primarily stability-providing, anchoring junctions are the adherens junctions, desmosomes and hemidesmosomes. They connect the cytoskeleton between neighboring cells, and anchor it to the extracellular matrix. Communicating junctions are built by gap junctions, which allow the passage of cytosolic components from cell to cell (Levendoski *et al.* 2014).

2.2.1 Location and structure of tight junction proteins

On ultrastructural level, tight junctions build a band around the apical border of epithelial cells, interconnecting them in a meshwork of strands. In general, strand organization is tighter on the apical-most border, followed by less organized medial and basal strands (Farquhar and Palade 1963; Schneeberger 1980; Staehelin 1973). By comparison of different tissues, the kissing points of membrane contacts vary in morphology and number (Friend and Gilula 1972; Claude and Goodenough 1973) and show a variable permeability to solutes and ions (Fromter and Diamond 1972). Among the main protein families described to form the tight junction complexes, namely the TAMPs, MAGUK-family and claudins, the tetraspan family of claudins are the essential barrier forming proteins, as demonstrated in transfection studies (Furuse *et al.* 1998b, Furuse *et al.* 2001). The 20-25 kDa claudin-proteins determine the tissue specific barrier properties, as they differ in quantity and composition of their over 60 members in vertebrates – 27 known in humans – among different epithelia (Kolosov *et al.* 2013, Markov *et al.* 2010, Mineta *et al.* 2011). A structural analysis of a single claudin molecule was performed recently, revealing a ß-sheet containing architecture of two extracellular loops, anchored *via* four left handed transmembrane helices (Suzuki *et al.* 2014). While the fundamental unit is a dimer (Van Itallie *et al.* 2011), the single claudin molecule would be about 10 nm in size, due to freeze fracture measurements (Anderson 2001). Most claudins can interact with ZO-proteins *via* a carboxyl terminal PDZ-binding motif (Itoh *et al.* 1999), but organization in homo- and heterodimers of different claudins (Piontek *et al.* 2011), and molecular structure of the extracellular loops promoting cis and trans configuration suggest a model of defined claudin clusters (Furuse *et al.* 1999; Markov *et al.* 2015). However, since splicing of single claudins can alter their location and function (Günzel *et al.* 2009), further research is needed to elucidate possible claudin clusters and their physiological meaning.

Members of the TAMPs family of proteins, namely occludin, tricellulin and MarvelD3, do not form tight junction strands by themselves, but modulate strand architecture in combination with claudins, and - in case of occludin - can form transcellular dimers (Cording *et al.* 2013). Occludin knockout mice are vital and develop normal barrier properties but show diverse abnormalities in tissue histology and reproduction (Saitou *et al.* 2000), which supports the model of occludin in a regulatory function. On the molecular level, phosphorylation promotes a recruitment of occludin into the tight junction (Wong 1997), and a binding sequence on the carboxyl terminal domain allows an interaction with ZO-1 (Li *et al.* 2005).

ZO proteins are scaffolding proteins, which have binding sequences for occludin and diverse claudins. They promote a link to the actin cytoskeleton of the cell (Fanning *et al.* 1998; Itoh *et al.* 1999), which explains their location below the tight junction strands, observed by electron

microscopy for ZO-1 (Stevenson *et al.* 1986). As members of the MAGUK family, they show a sequence with homology to guanylate kinase (Willott *et al.* 1993), and homologous dimerization of ZO-1 or heterologous with ZO-2 and ZO-3 was observed *via* the second of three PDZ binding domains (Fanning *et al.* 2007). Although the role of ZO-1 in the tight junction is likely not fully elucidated, one important function is the direction of claudins and occludin to their appropriate location (Fanning *et al.* 2006).

2.2.2 Barrier function and tight junction associated diseases

Several physiological functions are known to be fulfilled by tight junction proteins. First, the gate function represents the organ- and tissue specific barrier and permeability mediating function of tight junction proteins, which primarily is determined by the family of claudins (Amasheh *et al.* 2011, Mandel *et al.* 1993). In addition, a fence function (Mandel *et al.* 1993; van Meer and Simons 1986), signaling- (Beeman *et al.* 2012) and mechanical adhesive functions (Pitelka *et al.* 1973) are other known functions of tight junction strands. In their gate function, tight junction proteins control the paracellular space, that means the passage of ions (Amasheh *et al.* 2002), solutes (Watson *et al.* 2001), proteins (Zhuang *et al.* 2015), water (Rosenthal *et al.* 2010) and bigger particles as *e.g.* bacteria (Veshnyakova *et al.* 2012), cells of the immune system (Lin *et al.* 1995) and cancer cells (Brennan *et al.* 2010). Depending on strand morphology and claudin composition, “tight” and “leaky” tight junctions can be distinguished in epithelial barriers (Claude and Goodenough 1973; Furuse *et al.* 2001). On the molecular level, “tightening” and “permeability mediating” claudins can be distinguished, as well as claudins with ambiguous functions (Amasheh *et al.* 2011, Markov *et al.* 2015).

Knockout models played an important role in better understanding the function of single tight junction molecules. Therefore, it is known that knockouts of occludin and tricellulin are compatible with life and even develop physiological intestinal barrier properties (Kamitani *et al.* 2015; Saitou *et al.* 2000; Schulzke *et al.* 2005), while knockout of ZO-1 leads to embryonic death (Katsuno *et al.* 2008). However, in mammary epithelial cells, occludin was shown to mediate apoptosis after its dislocation from the tight junction (Beeman *et al.* 2009). Moreover, it additionally plays a key role in apoptosis after claudin disruption (Beeman *et al.* 2012). Claudins are the critical proteins concerning permselectivity of epithelial barriers, and deficiency of single claudins could elucidate their specific function. This was first accomplished for claudin-1 in mice, which died one day after birth because of dehydratation, due to massive water loss through the skin (Furuse *et al.* 2002; Sugawara *et al.* 2013). Other examples for claudin deficiency and hereditary or acquired claudin related diseases are: a reduced reabsorption of sodium in kidneys of claudin-2 lacking mice (Muto *et al.* 2010), the

crucial role of endothelial claudin-5 in maintaining the blood brain barrier (Nitta *et al.* 2003), claudin-16 in calcium and magnesium transport function (Kausalya *et al.* 2006; Nadarajah *et al.* 2014) and claudin-3 and -4 as target molecules for clostridium perfringens enterotoxin (Veshnyakova *et al.* 2012). Both, mice with claudin-3 deficiency and mice with claudin-4 deficiency are reported to be viable and fertile (Fujita *et al.* 2012; Kage *et al.* 2014; Kerr *et al.* 2015; Schröder 2013). However, claudin-4 knockout mice were more susceptible to lung injuries (Kage *et al.* 2014) and after several months developed a lethal hydronephrosis due to urothelial obstruction (Fujita *et al.* 2012). On the molecular level, claudin-4 is known to provide paracellular Cl^- channels, and it acts as a barrier against sodium (Hou *et al.* 2010; Van Itallie *et al.* 2001). Instead, claudin-3 has a barrier providing function for ions of both charge, and additionally acts as a barrier against uncharged molecules (Milatz *et al.* 2010).

2.3 Hormone-dependence of the mammary gland development

It is well established knowledge, that mammary gland tissue undergoes several phases of development until it serves its physiological function to nurse offspring – most of whom occur after birth – namely mammogenesis, lactogenesis and galactopoiesis (Tucker 2000), followed by a phase of involution after weaning (Borellini and Oka 1989). During the embryogenesis, mammogenesis is independent from hormone-presence but is induced by mesenchymal control. Conversely, it can be inhibited by androgen production in male embryos (Borellini and Oka 1989). Postnatal, two phases of allometric growth follow in female mammary gland development: First, during puberty, elongation and branching of the ductal system occurs. As demonstrated in mice, this process is primarily initiated by estrogen (Shyamala and Ferenczy 1984). During pregnancy, ovarian steroids mediate lobuloalveolar formation in preparation of lactation, which is accompanied by another phase of allometric growth (Borellini and Oka 1989). However, hormones produced by the pituitary gland are also required in mammogenesis, namely prolactin and growth hormone (Borellini and Oka 1989; Tucker 2000), as elucidated for prolactin in prolactin receptor knockout-mice (Brisken *et al.* 1999). Prolactin is known to play a main role in the physiology of the mammary gland, not only in mammogenesis, but also during lactogenesis (Shaar and Clemens 1972). Moreover, in combination with hydrocortisone and insulin, prolactin is able to induce the production of lactation specific proteins, *in vitro* (Juergens *et al.* 1965). *In vivo*, a dropdown of progesterone – as physiological for the time of parturition – was shown to induce lactogenesis (Liu and Davis 1967). However, galactopoiesis is independent from progesterone, as it is unable to inhibit an established lactation (Herrenkohl 1972). Even ruminants milked before partum can produce milk of normal composition, indicating other mechanisms than the dropdown of progesterone to be determining for the initiation of

galactopoiesis (Linzell and Peaker 1974). While mammary gland development in the living animal requires an orchestrated action of different hormones, and is a complex, not yet fully understood process, several *in vitro*-models have been established, using key hormones to induce lactation specific differentiation, such as prolactin and cortisol (Juergens *et al.* 1965, Ono and Oka 1980, Schneider and Shyamala 1985).

2.3.1 Mammary gland epithelial cell model HC11

The use of cultured monolayers as a model for epithelial tissue is a common praxis in tight junction research, as *e.g.* performed with MDCK cells (Amasheh *et al.* 2002), HT-29/B6 cells (Amasheh *et al.* 2010) and Caco-2 (Amasheh *et al.* 2005). Among established cell lines isolated from the mammary gland epithelium, HC11 is characterized by spontaneous immortalization, unlimited passage number, murine female origin and response to hormone induction, *i.e.* prolactin-dependent production of the milk protein ß-casein (Ball *et al.* 1988, Perotti *et al.* 2009, Williams *et al.* 2009). HC11 cells were clonally derived from the COMMA-1D cell line (Ball *et al.* 1988), which originally was isolated from mid-pregnant mice of the mouse subspecies BALB/c (Danielson *et al.* 1984). **Fig. 3** depicts the histological characteristics of this cell line. In HC11 cells, an upregulation of occludin was induced by prolactin and glucocorticoids (Stelwagen *et al.* 1999), and ZO-1 expression can also be enhanced by glucocorticoids in mammary epithelial cells, *in vitro* (Singer *et al.* 1994). Other mammary epithelial cell lines derived from mice are *e.g.* tumor cell lines as 66, 410.4 and D2A1 (Miller *et al.* 1989), or spontaneous immortalized cell lines with limited or no described ability to differentiate, as *e.g.* NMuMG (Owens *et al.* 1974, Sizemore and Cole 1982) and COMMA-1D, including its derivations CDNR2 and CDNR4 (Danielson *et al.* 1984, Danielson *et al.* 1989). Regarding cows, several bovine mammary epithelial cell lines have been described: The ET-C cell line exhibits characteristics of both, mammary epithelium and myoepithelial cells, and therefore may have a stem cell nature (Zavizion *et al.* 1995). MAC-T cells are transfected with SV-40 large T-antigen and show prolactin-induced casein secretion (Huynh *et al.* 1991). A low synthesis of milk proteins can be detected in BME-UV cells, which also were immortalized using the SV-40 (Zavizion *et al.* 1996). A study comparing the MAC-T and BME-UV1 cell line found the latter one to show more characteristics of mammary alveolar epithelium (Arévalo-Turrubiarte *et al.* 2016). Zhao *et al.* used human telomerase reverse transcriptase to immortalize bovine mammary epithelial cells (Zhao *et al.* 2010). Very recently, two more cell lines with secretory properties were established, namely CMEC-H (Hu *et al.* 2016) and hTBME (Li *et al.* 2018).

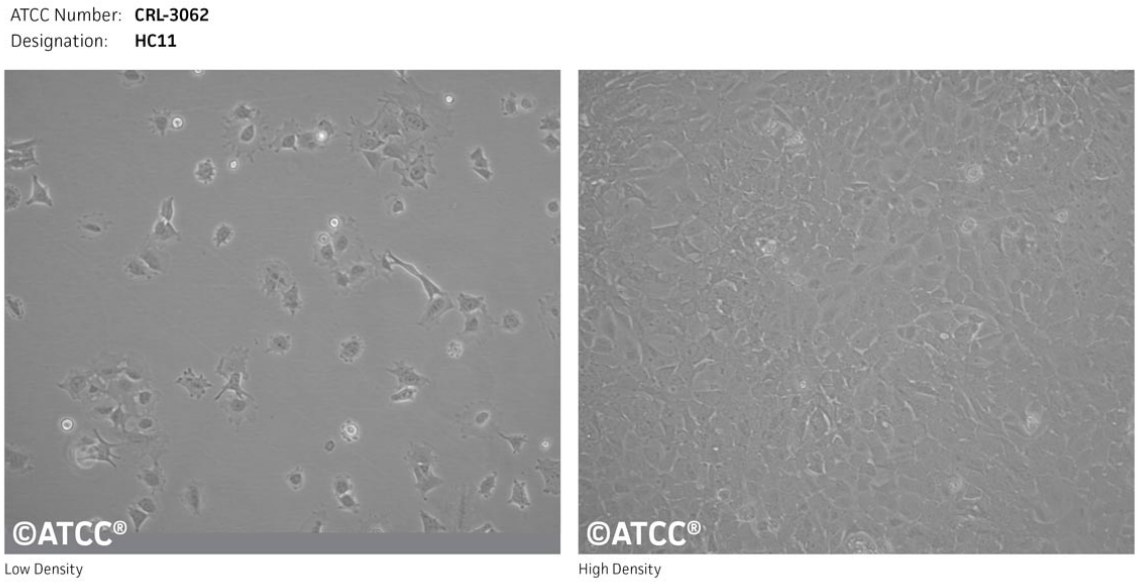

Figure 3: Light microscopy of growing (left) and confluent (right) HC11 cells
Cultured HC11 cells show epithelial-like morphology and form adherent monolayers. They are used as *in vitro*-model to study mammary epithelial cell proliferation, differentiation and signal transduction (from ATCC®):
https://www.lgcstandards-atcc.org/~/media/Attachments/A/1/7/6/CRL-3062%20Low%20High.ashx

2.3.2 Barriology of the mammary epithelium during differentiation

Since milk composition differs significantly from that of blood plasma, as *e.g.* milk contains high amounts of lactate and milk proteins and low concentrations of sodium and chloride in comparison to plasma (Barry and Rowland 1953, Cerbulis and Farrell 1975), the transepithelial barrier of mammary epithelial cells has to be very tight during galactopoiesis. This is represented on electrophysiological level by a potential difference of -35 mV, maintained between the two compartments (Berga 1984). Around parturition, with the onset of lactation, milk composition changes in its concentrations of lactose, ions and proteins (Linzell and Peaker 1974). These processes require a tightening of the epithelial barrier during lactogenesis, as represented on molecular level by changes in tight junction morphology and composition (Itoh and Bissell 2003, Kobayashi *et al.* 2016, Morgan and Wooding 1982, Nguyen *et al.* 2001). Moreover, changes in mammary epithelial junctions not only occur in tight junctions, but also affect adhesive junctions and desmosomes, which are rarely detectible after the differentiation process into the lactating stage (Pitelka *et al.* 1973). It can be shown that barrier properties of mammary epithelial cells change from a leaky to a tight status during differentiation, by the fact that permeability for different substances decreases significantly in that process, as demonstrated *e.g.* for albumin and sucrose

(Nguyen *et al.* 2001). Conversely, with cessation of lactation and the beginning involution of the mammary gland, tight junctions become leaky again (Fleet and Peaker 1978), a process which is promoted by serotonin (Hernandez *et al.* 2011).

Processes of adaptation, including claudin expression pattern and dynamic remodeling within the tight junction, are called claudin switching. They occur during differentiation, and along organs with specialized areas like the intestine (Capaldo and Nusrat 2015). During the established lactation, claudin-1, -2, -3, -4, -5, -7, -8, -15, -16 and occludin are described to be expressed in the mammary gland of mice, as detected with PCR and Western blotting from mammary gland lysates (Markov *et al.* 2012). In differentiated HC11 cells, ZO-1, ZO-2, occludin and claudin-1, -3, -4, -5, -7, -8, -15 and -16 were identified, while claudin-2, -10, -14 and -18 were not detectible *via* Western blot analysis (Reiter *et al.* 2006). Baumgartner *et al.* (2017) analyzed the mRNA expression of several claudins from pregnancy day 11.5 to lactation day 9 in mammary glands of mice. They consider claudin-3, -7 and -8 to be the most important claudins in mammary epithelium during an established lactation. While claudin-7 was expressed constantly at a high level, claudin-8 increased more than 25-fold with the onset of lactation. Claudin-3 decreased around parturition, but nevertheless showed significantly high expression levels. A dropdown of claudin-4 contemporaneous with the progesterone downfall at pregnancy day 18.5 resulted in almost no signals for claudin-4 during lactation (Baumgartner *et al.* 2017). Immunofluorescent staining revealed a localization of claudin-3 and claudin-4 in the apical-most regions and in the lateral membrane at pregnancy day 17. However, with the onset of lactation, claudin-4 disappeared successively from the cells and claudin-3 was predominantly colocalized with occludin within the tight junctions (Kobayashi and Kumura 2011).

During the established lactation, periods of milk stasis can change the barrier properties and cause a tight junction leakage as a short-term effect (Stelwagen *et al.* 1994, Stelwagen *et al.* 1997). On the tight junction protein level, Markov *et al.* found changes in the claudin composition after 20 hours of milk stasis in mice, which suggests a sealing process, as depicted in **Fig. 4** (Markov *et al.* 2012). However, *in vivo* studies are limited in their ability to determine the involvement of different aspects in the complex physiological processes of a living animal. It was shown *in vitro*, *e.g.* for the intestine, that milk (Radloff *et al.* 2017) and single milk components (Dittmann *et al.* 2014) can affect epithelial barrier properties. Moreover, barrier properties of mammary epithelial cells were also influenced by milk components. In particular, HC11 cells could be stimulated by the hyperimmune milk factor (Stelwagen *et al.* 1998). Shamay *et al.* found casein hydrolysates as a factor responsible for tight junction disruption, which might have importance during the physiological process of involution (Shamay *et al.* 2003). Therefore, to analyze mammary epithelial barrier properties under the influence of pressure generated by milk accumulation – isolated from effects

caused by milk components – an *in vitro* approach was considered appropriate, using a customized Ussing chamber to apply hydrostatic pressure.

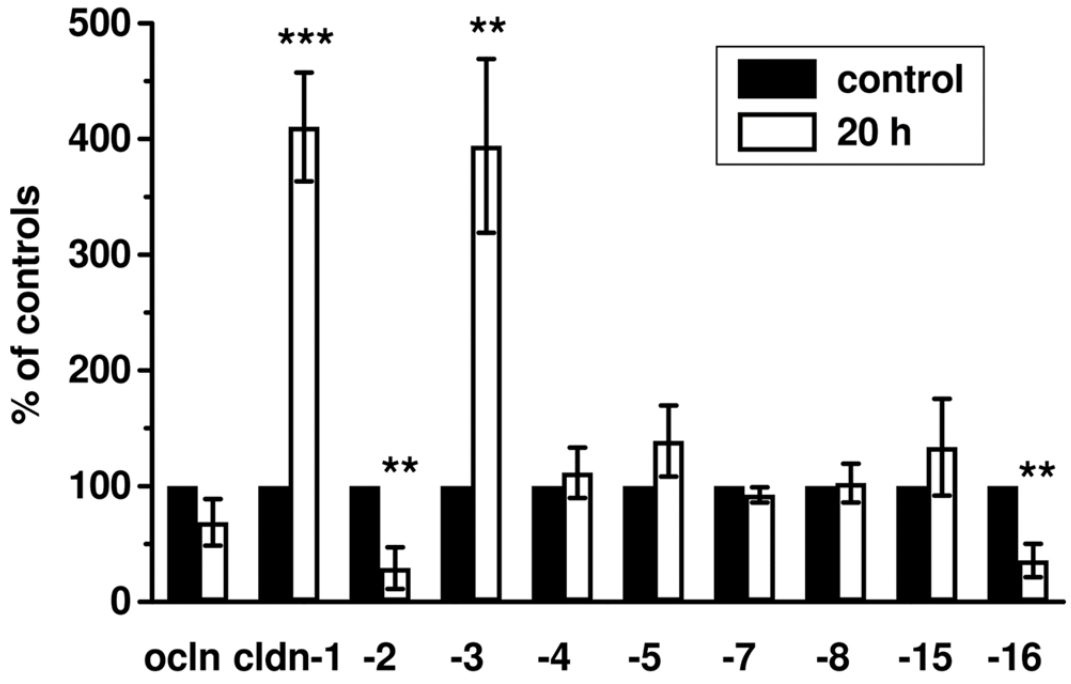

Figure 4: Claudin expression after 20 hours of milk stasis in mice mammary gland tissue, *in vivo*

Densitometric evaluation of Western blot analysis revealed a decrease of pore forming claudin-2 and -16, together with an increase of claudin-1 and -3, which are generally known as barrier forming proteins ($n = 4–7$, respectively, $^{**}p < 0.01$, $^{***}p < 0.001$; from Markov *et al.* 2012).

2.4 The Ussing chamber technique

Since Hans H. Ussing 1951 developed an apparatus to simultaneously measure chemical and electrophysiological parameters of epithelial transport mechanisms in frog skin (Ussing and Zerahn 1951), the Ussing chamber represents a widely distributed and often employed electrophysiological tool. Whereas the principle of active transport over membranes – against the electrical and chemical gradient – was demonstrated before (Ussing 1948), and the practice to measure current over a short-circuited frog skin was also established (Francis and Pumphrey 1933), the Ussing chamber allowed the first prove that an active transport of sodium was responsible for the difference in the electric potential across epithelial membranes (Ussing and Zerahn 1951). The experimental setup was built of two separate compartments containing the same solution and divided by an epithelial sample tissue. Using

a connected power source, the transepithelial electrical potential was short-circuited, and thus it was possible to measure the transport of a radioactive tracer ion and electrical current simultaneously (Ussing and Zerahn 1951). The schematics of the Ussing chamber apparatus are shown in **Fig. 5**.

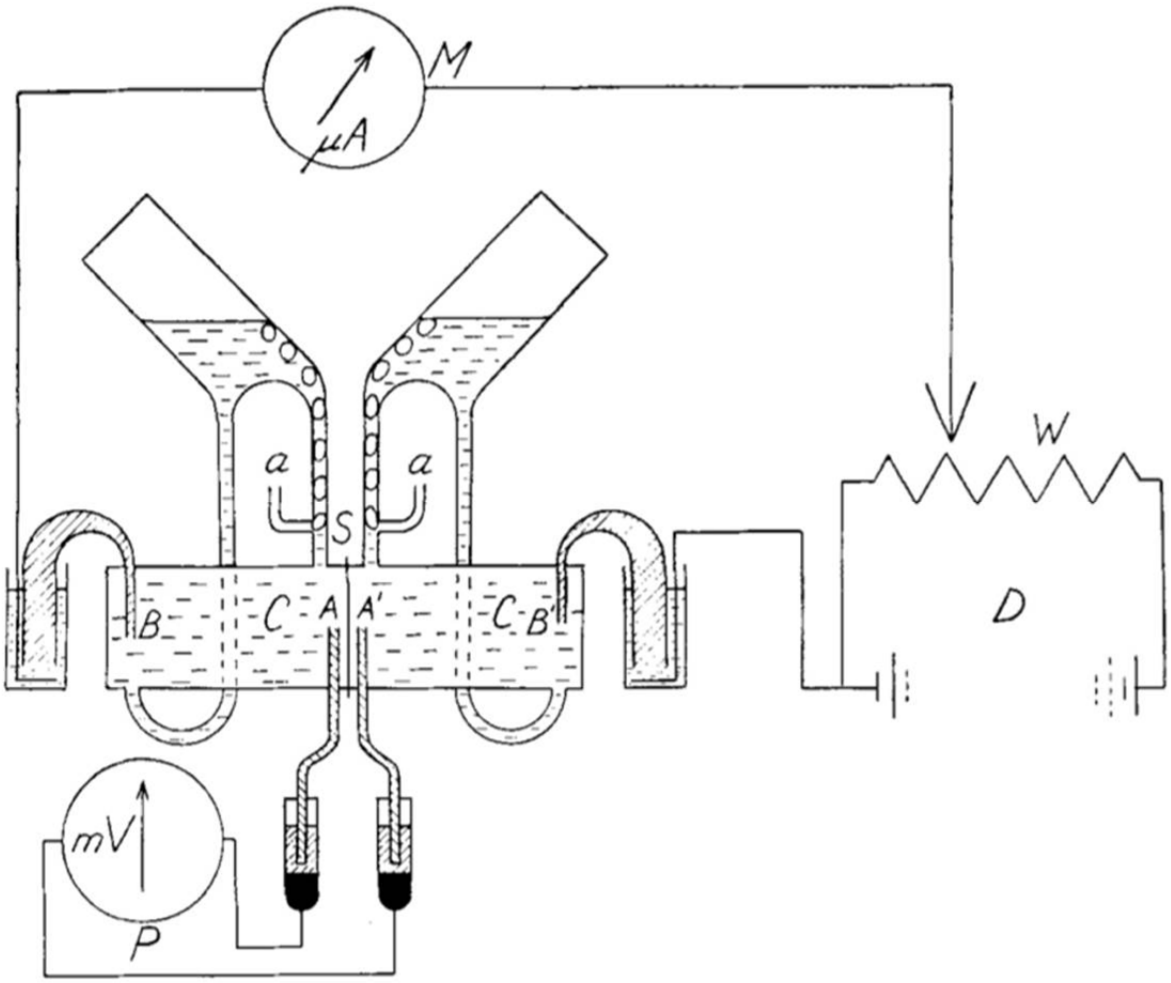

Figure 5: Schematic overview of the Ussing chamber apparatus

The experimental tissue (S) is inserted between two half chambers (C), filled with experimental buffer. Buffer circulation and oxygenation are controlled using a gas supply (a). Near the tissue, voltage sensing electrodes (A, A´) are placed and connected to a volt measuring device (P). A current source (D) and a potential divider (W) are used to short-circuit the voltage to zero by applying current through electrodes (B, B´), which are placed in the chamber. With an ampere meter (M) the resulting current I_{SC} – caused by active transport – is measured (Hamilton 2011; reproduced from Ussing and Zerahn 1951).

Subsequent studies revealed several transport principles in frog skin, *e.g.* that transport of Cl^- is passive under most conditions (Johnsen *et al.* 1952; Koefoed-Johnsen *et al.* 1952), and in

contrast, sodium and potassium are transported actively in a linked antiport, located at the basolateral side of the epithelium (Koefoed-Johnsen and Ussing 1958; Bricker *et al.* 1963). Therefore, the Ussing chamber technique proved to be the basis for the definition and analysis of active and passive transport over epithelia. Later, impedance analysis allowed the discrimination between epithelial and subepithelial barrier function (Gitter *et al.* 1998), followed by the discrimination between transcellular and paracellular barrier function by the same group (Gitter *et al.* 2000). Based on these requirements, the first paracellular channel was identified (Amasheh *et al.* 2002), and barrier analysis was employed regarding local characteristics of single organs, as accomplished *e.g.* for the intestine (Markov *et al.* 2010, 2016). Furthermore, addition or removal of specific components to the experimental solution is a wide spread technique, as successfully practiced *e.g.* with hormones (Amasheh *et al.* 2004), microbial toxins (Markov *et al.* 2014), proinflammatory cytokines (Barmeyer *et al.* 2004), and secondary plant metabolites (Amasheh *et al.* 2010, 2012).

To meet the different challenges in barrier and transport research, several improvements and modifications were performed on Ussing's original setup, as *e.g.* the possibility to mount cultured cells grown on permeable supports (Misfeldt *et al.* 1976) and the design of special micro-chambers to analyze very small samples of < 1 mm in diameter (Mall *et al.* 1998). Nowadays, two main types of chambers are in general use: a circulating chamber and a perfusion chamber. The circulating chamber works similar to Ussing's construction and is a commercially available device. The main difference to the perfusion chamber lays in the part containing the experimental solutions, which in the circulating chamber is located in a double-walled U-shaped glass reservoir. Circulation and oxygenation of the buffer are maintained by gas bubbles and temperature is regulated using warmed water between the two walls of the reservoir. In perfusion chambers, experimental solutions are stored in separate reservoirs and continuously applied to the chambers, thus allowing to change buffer composition during the experiments and to minimalize hydrostatic pressure (Hug 2002, Li *et al.* 2004). Not just the chamber was improved since 1951, but also the electrical circuitry has developed.

In principle, electrical parameters can be calculated using Ohm's law:

$$R = \Delta V / \Delta I$$

Applying Ohm's law to the electrophysiological parameters of an Ussing chamber setup results in the following equation:

$$R_T = V_T / I_{SC}$$

Ussing used "voltage clamping", *i.e.* he short circuited the voltage to keep V_T = 0 mV and measured the resulting current I_{SC}. Applying Ohm's law to an epithelial tissue, R_T can be calculated if V_T and I_{SC} are known. While this is still an often-used technique, other

approaches have been successfully established since then, *e.g.* “current clamping” and the analysis of complex parameters like impedance and capacitance (Herrmann and Turner 2016, Hug 2002, Li *et al.* 2004).

In addition to the perfusion chamber, which is designed to minimalize hydrostatic pressure, different approaches have been published describing the analysis of electrophysiological parameters of the epithelial barrier under increased hydrostatic pressure conditions. Hans H. Ussing himself modified his apparatus to examine unilateral hydrostatic pressure effects on the inside of isolated frog skin (Voute and Ussing 1970). The setup was designed as a perfusion-type chamber, allowing unilateral pressure application up to 90 cm (~ 9 kPa) and simultaneous observation with a microscope from the other side of the tissue (Ussing 1965; Voute and Ussing 1970). Small hydrostatic pressure differences about 5 cm (~ 0.5 kPa) were studied by Eldrup *et al.* (1982) in rabbit gall bladders, using a modified Ussing chamber setup. Comparable setups were employed on pleural and on tracheal tissue of dogs (Payne *et al.* 1988; Kondo *et al.* 1992), and on rabbit urinary epithelium (Wang *et al.* 2003), applying hydrostatic pressure differences up to 20 cm (~ 2 kPa). An unmodified Ussing chamber was used to apply small pressure differences by removal of bathing solution on rabbit urinary bladders (Ferguson *et al.* 1997). Recently, studies using small hydrostatic pressure differences of 5 and 10 cm (~ 0.5 and 1 kPa) were performed on lung epithelial tissue in customized Ussing chambers (Bogdan *et al.* 2008; Richter *et al.* 2014; Vitzthum *et al.* 2015). However, all these approaches investigated pressure differences, and therefore used Ussing chamber setups designed to apply hydrostatic pressure on just one side of the tissue. Additionally, if a setup is designed to be used in combination with substances added to the bathing solution, as *e.g.* blockers or tracers, stable conditions are required concerning buffer characteristics and distribution of additives, which has to be confirmed during establishment.

Chapter 3: Aims and Objectives of the Thesis

As the natural barrier between corporal side and environment, epithelial tissue needs mechanisms for both, to maintain the epithelial barrier and to regulate a controlled exchange of substances. In lactating mammary gland tissue, produced milk is collected in the mammary gland and significantly rises the intraglandular pressure. Accumulation of milk has been shown to have several effects on the mammary epithelial tissue, *in vivo*. On the histological level, flattening of epithelial cells has been described, together with changes in tight junction composition on molecular level (Markov *et al.* 2012).

However, milk components are also known to induce changes in tight junction composition on epithelial tissue, as described *e.g.* for the intestine. There were no studies available which differentiate between pressure induced effects and milk induced effects on the barrier properties of mammary epithelial monolayers.

The aim of the thesis was to further investigate the mechanisms of mammary gland epithelium to maintain the epithelial barrier (I) despite high intraglandular pressure during milk accumulation and (II) during negative pressure caused by suckling or milking. The main goal was to dynamically analyze effects of hydrostatic pressure on the barrier function of mammary epithelial monolayers without the presence of milk components, *in vitro*.

The following specific questions were addressed concerning hydrostatic pressure effects on barrier properties of the mammary epithelial cell line HC11:

- electrophysiological parameters during application of increased basal pressure
- structural integrity of the monolayers after basal pressure challenge
- electrophysiological parameters during bilateral pressure application
- structural integrity of the monolayers after application of bilateral pressure
- quantity of single tight junction proteins after bilateral pressure incubation

Increasing basal pressure was applied in long and short time steps. In addition to the different pressure setups, the influence of varied cultivation times of the cell monolayers and differentiation state were taken into account.

Prerequisite for these investigations was an appropriate tool to dynamically analyze electrophysiological parameters of epithelial monolayers during pressure changes. To acquire this, the first goal was to establish a modified Ussing chamber setup to meet the technical requirements, accordingly.

In detail, the following aims were defined:

(1) Establishment of a modified Ussing chamber which can be used with hydrostatic pressure setups from 1 kPa up to 10 kPa. To accomplish this, a normal Ussing chamber setup was modified with a tube system to apply hydrostatic pressure, regulated by a syringe and monitored by a manometer. To confirm stable conditions, fluorescein distribution and pH were tested four times in a time interval of 15 minutes, using a setup of 10 kPa bilateral hydrostatic pressure.

(2) Analyses of increasing negative pressure-effects to HC11 monolayers, cultivated for 7 and 14 days. Two setups of a basal hydrostatic pressure challenge were performed, using 5 and 30 minutes between the pressure applications. Processes of readjustment to the normal pressure level were analyzed in a conclusive equalization step, and electrophysiological studies were complemented by immunohistochemistry.

(3) Investigation on the effects of bilateral pressure incubation on 14 day-cultivated HC11 monolayers, in dependence of differentiation by prolactin and dexamethasone. Additional experiments were performed using barium chloride as a blocker for a possible chloride secretion. In addition to the Ussing chamber experiments, tight junction proteins were analyzed employing immunohistochemistry, and quantification of these proteins was done using the Western blot technique.

Chapter 4: Basolateral pressure challenges mammary epithelial cell monolayer integrity, *in vitro*

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Katharina S. Mießler, Constanze Vitzthum, Alexander G. Markov, Salah Amasheh

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Chapter 5: Hydrostatic pressure incubation affects barrier properties of mammary epithelial cell monolayers, *in vitro*

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You have to read this part online.

Chapter 6: Discussion

Hydrostatic pressure affects mammary epithelial barrier function, *in vitro*

Goal of the first part of this thesis was (I) to successfully establish a technique for the dynamical analyses of mammary epithelial barrier properties under hydrostatic pressure changes – isolated from the presence of milk components, and (II) to test the hypothesis if increasing hydrostatic pressure, applied to the basal side of the epithelium, is able to affect mammary epithelial monolayers, *in vitro*. To achieve these goals, a conventional Ussing chamber setup (Hug 2002, Li *et al.* 2004) was modified to dynamically change buffer quantity during experiments, and thus induce hydrostatic pressure in a range from 1 to 10 kPa on the basal, apical or both sides of the epithelium. The setup was evaluated regarding its ability to maintain constant pH and distribution of fluorescein. A schematic view of the modified Ussing chamber is shown in **Fig. 1** (p. 22), supplemented by the results showing that pH and fluorescein distribution remained constant during the maximal pressure setup of bilateral 10 kPa.

Using this customized Ussing chamber setup, HC11 monolayers were exposed to five steps of basal hydrostatic pressure, increasing from initial 1 kPa on both sides to +5 kPa basal, which results in 1 kPa apical and 6 kPa basolateral. The pressure increase was followed by a conclusive step of pressure equalization to 1 kPa for five minutes. Epithelial ability to adapt to basal pressure challenges was taken into account. Therefore, incubation intervals of 30 and 5 minutes were chosen, and HC11 cells were used after 7 and 14 days of cultivation, resulting in four experimental series. Since HC11 monolayers cultivated for 7 days did not successfully withstand a 5 minute-interval of increasing basal hydrostatic pressure, the remaining three experimental series could be used to analyze the effects of a pressure challenge from basal, in detail. All three series of experiments revealed a strong reduction of R_T initiated by the first basal pressure application, and a successive increase of I_{SC} during the experimental time. Both, the reduction of R_T and the increase of I_{SC} were partially reversible after pressure equalization. The electrophysiological changes are graphically depicted in **Fig. 2** (p. 24) and **Fig. 3** (p. 25). Despite its strong effects on electrophysiological barrier properties, basal hydrostatic pressure did not disturb the monolayer integrity, as verified by immunofluorescence histology. Since desmosomes and adhesive contacts disappear during the onset of lactation, the tight junction determines epithelial cell integrity in lactating alveoli (Pitelka *et al.* 1973). Immunofluorescence staining of ZO-1, occludin and claudin-3 was performed in HC11 monolayers cultivated for 14 days, after basal pressure incubation with a 5 minute-interval – confirming monolayer integrity and revealing no differences in concentration or distribution between control and experiment, see **Fig. 4** (p. 25).

With establishment of the modified Ussing chamber technique – and based on the finding that basal hydrostatic pressure influences electrophysiological barrier properties in HC11 monolayers – goal of the second part of this thesis was to test if bilateral pressure incubation can induce changes in barrier properties of HC11 monolayers – on electrophysiological and on tight junction protein level.

Experiments were carried out using HC11 monolayers cultivated for 14 days, since 7 day-cultivated monolayers turned out to be less able to adapt to hydrostatic pressure in the first study. To further evaluate effects of maturing processes taking place at the onset of lactation (Baumgartner *et al.* 2017, Kobayashi and Kumura 2011), HC11 cells were differentiated using hormone induction in a parallel approach. Both, HC11 monolayers with and without hormone treatment were exposed to 10 kPa bilateral hydrostatic pressure for a time interval of 4 hours. This incubation led to a decrease of I_{SC} in differentiated monolayers, which could be observed after 2 hours and which lasted until the end of the experiments. Mechanisms responsible for the decrease in I_{SC} were further analyzed employing 1 mM barium chloride as established blocking substance for chloride secretion (Kreusel *et al.* 1991, Silva *et al.* 1986). Addition of the blocking substance successfully inhibited the decrease of I_{SC} after 2 hours but did not last until the end of incubation time, indicating a relevant role of chloride secretion in the decrease of I_{SC}, which may be complemented by other mechanisms. On the molecular level, changes in barrier properties were reflected by changes in ZO-1, showing an upregulation in differentiated cells, but a downregulation with addition of barium chloride. Surprisingly, a decrease of occludin was observed in undifferentiated cells *via* Western blot quantification. The decrease in occludin concentration was also visible in immunofluorescent stained monolayers, whereas no changes in R_T and I_{SC} could be observed in these cells – with or without the addition of barium chloride. However, high deviations were measured in the I_{SC} of undifferentiated cells with addition of barium chloride, which may explain the unspecific outcome. For graphical depiction of I_{SC} see **Fig. 1** (p. 36), and for Western blot analysis see **Fig. 2** (p. 36).

Approaches to analyze mechanical forces in barriology

Mechanical forces and their effects on different physiological models have been investigated using diverse approaches, depending on the character of the analyzed force, the experimental tissue, available experimental tools and the leading question. Aside from pressure, other artificial physical stimulations were employed on different physiological models, *e.g.* the influence of vibration on cell adhesion (Ito *et al.* 2011) and effects of stretching in cardiomyocytes (Mihic *et al.* 2014). One prominent example may be the effect of shear stress on endothelial tissue, which has been investigated employing different systems,

as *e.g.* flow chamber experiments (Brakemeier *et al.* 2003, Partridge *et al.* 2007) and the cone-and-plate apparatus (Spruell and Baker 2013), and regarding different endothelial models, *e.g.* of arterial (Partridge *et al.* 2007) and venous origin (James *et al.* 2011). Whereas research in thrombosis and blood stasis led to several improvements of *in vitro*-techniques for the analysis of shear stress, including the development of microfluid devices (Zhang and Neelamegham 2017) and a hemodynamic simulator capable to induce stretch, shear and pressure forces (Berardi and Tarbell 2009), studies of pressure conditions in mammary gland tissue were often designed as *in vivo*-experiments (Alex *et al.* 2015, Davis *et al.* 1998, Markov *et al.* 2012, Stelwagen *et al.* 2013). A reason for the dominance of *in vivo*-studies in that field in comparison to other mechanical forces may be, that available tools to apply vibration, stretch or shear stress can be also applied in epithelial models (Ito *et al.* 2011, Mihic *et al.* 2014, Partridge *et al.* 2007), whereas approaches to apply hydrostatic pressure on epithelial tissue and to simultaneously analyze barrier properties in real time are limited (Ferguson *et al.* 1997, Voute and Ussing 1970, Wang *et al.* 2003), and do not include bilateral pressure. Studies simulating intraglandular pressure *in vitro* are scarce, and dynamical analyses of hydrostatic pressure effects on barrier properties in real time were not available for a mammary epithelial cell model until establishment of the modified Ussing chamber setup.

Three studies employing mammary epithelial cells in an Ussing chamber setup are available to date, none of which studying hydrostatic pressure effects. Experiments were carried out using three different mammary epithelial models, namely HME (Palmer *et al.* 2011), BME-UV (Quesnell *et al.* 2007) and primary cultured cells (Bisbee *et al.* 1979). The latter were cultivated on floating collagen gels, a method which could not be combined with cultivation on permeable supports, and therefore was not suitable in the current experimental design. Apart from modified Ussing chamber systems, other methods have been developed to apply hydrostatic pressure on epithelial or endothelial cells. Among the recently published approaches, a compressed gas cylinder was used to induce pressure up to 40 mmHg (~ 5 kPa) on endothelial cells, which were exposed to pressurization *via* compression of a fluid layer, and thus were challenged with hydrostatic pressure (Shin *et al.* 2012). Long term pressurization of 6 days with high hydrostatic pressure values of up to 100 kPa were performed, employing a hydrostatic bioreactor system to challenge urothelial carcinoma cells (Chen *et al.* 2014), and extremely high-pressure conditions of up to 200 000 kPa successfully inactivated fibroblasts and nevus cells (Morimoto *et al.* 2016). Pressure elevation during cell cultivation could be realized with aid of a gas-filled box-system, placed in a cell culture incubator (Xin *et al.* 2011). A lately developed two-chamber culture system for pressure incubation up to 60 cm water column (~ 6 kPa) can be used with cells seeded on permeable supports (Hagiyama *et al.* 2017, Yoneshige et al 2017), and could be combined with the

current setup if pre-pressurization during cultivation is needed in future experiments. Whereas some of these techniques may be suitable to apply the desired amounts of hydrostatic pressure to mammary epithelial cells, none of them provides the possibility to simultaneously analyze electrophysiological barrier and transport parameters – which can be accomplished using a modified Ussing chamber setup.

Consequently, the new established Ussing chamber setup provides a novel method for dynamical analyses of pressure effects on transport and barrier properties in a mammary epithelial cell model, *in vitro*. The system thereby allows different pressure setups between 1 and 10 kPa: (I) bilateral pressure, (II) unilateral pressure, and (III) the possibility to change pressure conditions during experiments.

In the first part of the thesis, relatively negative pressure is applied to mammary epithelial monolayers by increasing hydrostatic pressure on the basal side of the epithelium. Physiologically, mammary glands are exposed to negative pressure during the process of suckling (Rasmussen and Mayntz 1998), which in dairy farming is replaced by milking machines during mechanical milking (Enokidani *et al.* 2016, Rasmussen and Madsen 2000, Rasmussen *et al.* 1994). Challenging epithelial tissue with basal hydrostatic pressure most likely reflects this situation, although it is not a setup working with directly applied negative pressure. To date, there is no Ussing chamber setup available capable to apply direct negative pressure during experiments. Aside from the Ussing chamber technique, Nakadate *et al.* employed a pressure chamber designed to generate positive and negative pressure using the percussion of a pendulum. In this study, transendothelial electrical resistance was measured after pressurization, and cell culture inserts were therefore placed in an extra chamber (Nakadate *et al.* 2014). Comparable to an approach for pressure incubation during cell-cultivation using a gas filled box-system (Xin *et al.* 2011), a box with controlled inflow and outflow was used to generate culture conditions applying negative pressure (Liu *et al.* 2017). Another option for pressurized culture conditions is a negative pressure incubator, as employed to analyze effects of negative-pressure wound therapy on epithelial tight junctions (Hsu *et al.* 2010). Regarding negative pressure in combination with simultaneous measurement of electrophysiological barrier parameters, no current models are available accomplishing suitable approaches. Therefore, the current setup allows an approach to negative pressure application and can be used to study relatively negative pressure in different physiological experimental questions.

Mammary gland barriology: *in vivo versus in vitro*

Intramammary pressure conditions – including both: negative and positive pressure – were studied in different experiments *in vivo*, thereby often inducing pressure by an accumulation of milk fluid in the gland (Graf and Lawson 1968, Schmidt 1971, Tucker *et al.* 1961), and measuring negative pressure during suckling or milking processes in the living animal (Rasmussen and Mayntz 1998, Rasmussen *et al.* 1994). Research on mammary barrier properties challenged by pressure effects was also concentrated on animal experiments, as *e.g.* performed in cows (Stelwagen *et al.* 1997), goats (Ben Chedly *et al.* 2013, Stelwagen *et al.* 1994) and mice (Markov *et al.* 2012). The presence of many studies using *in vivo*-approaches can be explained by the lack of an appropriate *in vitro*-method. Nevertheless, several important insights were acquired due to *in vivo*-experiments, elucidating the mechanics of mammary tight junction regulation during lactation and accumulation of milk:

It is known that milk stasis in dairy ruminants' results in leaky tight junctions, associated with reduced milk secretion, declined mammary blood flow, an increase in plasma lactose and plasma alpha-lactalbumin, and higher amounts of sodium in milk (Stelwagen *et al.* 1994, Stelwagen *et al.* 1997). Experiments, employing the calcium chelator EGTA to induce disruption of the tight junction in mammary glands, also revealed increased plasma lactose levels, changed ion composition in milk, and declined milk secretion, but induced a temporary increase in mammary blood flow (Stelwagen *et al.* 1995). On the contrary, repeated periods of milk accumulation during once daily milking led to an adaption process, with an increase in plasma lactose only observed on the first day and showing a downregulation of alpha-lactalbumin and GLUT-1 on transcriptional level – the latter one being an uptake protein for the lactose precursor substrate glucose (Ben Chedly *et al.* 2011, Ben Chedly *et al.* 2013). In mice, 20 hours of milk accumulation were histologically associated with a higher amount of fat drops in cumulated milk, expanded alveoli, and a flattening of the alveolar epithelium. Western blot analysis of whole gland lysates showed signals for occludin and claudin-1, -2, -3, -4, -5, -7, -8, -15, and -16, thereby revealing an increase of tightening claudin-1 and -3, and a decrease of permeability mediating claudin-2 and-16 (Markov *et al.* 2012, see **Fig. 4** p. 13).

However, *in vitro*-studies further elucidated some aspect of epithelial barrier properties: Milk-borne components can influence mammary epithelial barrier properties, as shown for hyperimmune milk factor in HC11 cells (Stelwagen *et al.* 1998). In this study, hyperimmune milk factor inhibited an EGTA-induced reduction of R_T, increased the rate of recovery after EGTA-challenge, stimulated barrier formation of HC11 cells and inhibited cell growth. Additionally, milk components have been shown to affect different epithelia apart from mammary epithelial cells, as shown for hyperimmune milk factor in MDCK cells (Stelwagen *et al.* 1998). In the intestine, milk compounds are well known to affect barrier function, as

demonstrated *e.g.* for lactoferrin (Zong *et al.* 2016), glycine (Li *et al.* 2016), glutamate (Jiao *et al.* 2015) as well as caprate (Rosenthal *et al.* 2012) and laurate (Dittmann *et al.* 2014). Laurate induced a decrease of R_T and an increase in fluorescein permeability in human colonic HT-29/B6 cells (Dittmann *et al.* 2014). On the contrary, porcine milk was reported to increase R_T without a change in fluorescein permeability in the intestine of piglets (Radloff *et al.* 2017). These apparently contradictious findings highlight the relevance to further evaluate the role of single milk components in the barrier regulation of different epithelia.

The *in vitro*-findings illustrate a limitation of *in vivo*-approaches: *In vivo*, it is difficult to differentiate between barrier changes caused by increased pressure and other possible reasons, as *e.g.* milk-borne factors or hormone regulation. Additionally, animal experiments do not provide possibilities to evaluate epithelial barrier properties simultaneously with the controlled dynamical change of unilateral or bilateral pressure levels, as provided by the modified Ussing chamber. Moreover, molecular analyses of whole gland lysates, as often practiced after sacrificing experimental animals, is restricted in its possibilities to determine the specific cellular origin of detected molecules. This might have led to contradicting interpretations of findings in different studies. While Markov *et al.* detected claudin-1, -2, -3, -4, -5, -7, -8, -15, and -16 from control tissues in lactating mammary glands *via* Western blotting, Baumgartner *et al.* considered claudin-2, -6 and -11 not to be expressed in mammary gland tissue, and claudin-5 to be confined to mammary gland endothelium, as evaluated *via* RNA analysis (Baumgartner *et al.* 2017, Markov *et al.* 2012). *In vitro*-models, employing purified mammary epithelial cells or established cell lines, might be necessary to further elucidate the origin of those molecules. Therefore, with respect to the known limitations of animal experiments, and following guiding principles regarding ethical use of animals in testing, an *in vitro*-method for the analysis of hydrostatic pressure effects to epithelial barrier properties was established as part of this thesis. Accordingly, the method was used to further evaluate findings obtained from *in vivo*-experiments, questioning whether changes in tight junction expression observed after milk stasis – which was regarded a mechanism against back-leak of milk compounds (Markov *et al.* 2012) – can be induced by isolated application of mechanical forces, *in vitro*.

Electrophysiological reactions induced by hydrostatic pressure

Employing the established Ussing chamber technique, different time spans of hydrostatic pressure incubation were tested on HC11 monolayers, ranging from five minutes to 4 hours. Basal pressure studies were carried out using time intervals of 5 and 30 minutes for the single steps. Five steps of pressure application were performed, which resulted in total pressurization times of 25 minutes and 2.5 hours, respectively – followed by 5 minutes of

equalization time. Since times of suckling and milking are temporary challenges for the mammary gland (Rasmussen and Madsen 2000, Rasmussen and Mayntz 1998), focus of this study was the 5 minute-time-interval. Shorter time intervals of *e.g.* 1 minute could be technically feasible with the modified Ussing chamber setup but would require a quick adaptation of the epithelium to the changed pressure level, and therefore stable values of R_T and I_{SC} after a short time. Since negative pressure during suckling and milking occurs periodically, and with a duration of less than a minute (Rasmussen and Madsen 2000, Rasmussen and Mayntz 1998), shorter time intervals would be a possibility to further approach physiological conditions in future studies.

Bilateral pressure incubation was performed over a period of 4 hours after equilibration, because stable experimental conditions could be provided up to that time span, whereas longer setups led to a loss of R_T in HC11 monolayers under control conditions, as observed during the preliminary work. Pilot experiments were conducted focusing on a time span of 20 hours, as studied in mice after cessation of milk removal (Markov *et al.* 2012), but limitations concerning viability of HC11 monolayers became apparent during this approach, which led to a shorter incubation time of 4 hours. One possibility to increase viability during longer experiments could be the use of a refined experimental solution, providing optimized conditions for the intended time interval. Alternatively, a different mammary epithelial cell line may be employed for longer experimental setups. Both options may be explored during further research regarding long-term hydrostatic pressure incubations, *in vitro*.

The influence of maturing processes during cultivation and adaptive mechanisms during basal pressure incubation became apparent in a pilot study, using 7 day cultivated cells challenged by a 5 minute-time-interval. In these experiments, while R_T was approximately 0 $\Omega \cdot cm^2$, I_{SC} reached values over 1000 µA/cm² after the third application of 1 kPa basal pressure, and bathing solution passed from the basal to the apical side. This high permeable barrier condition was neither observed in experiments with monolayers cultivated for 14 days, nor in 7 day-cultivated monolayers incubated with a 30 minute-interval. The effect of differentiation due to hormone induction with prolactin and dexamethasone was not evaluated during studies with basal pressure but could be a promising option to further elucidate mechanisms concerning maturing, differentiation and time-dependent adaptation to increasing basal pressure in epithelial barrier properties. In general, an immediate strong decrease of R_T combined with a successive increase in I_{SC} was observed during basal pressure challenges in all experimental series, which may be interpreted as complementary processes of barrier protecting mechanisms. Regarding R_T, base line represents a limit of adjustment. While increasing I_{SC} may function as a second protective reaction, values over 1000 µA/cm² cannot be regarded physiological, but most likely reflect an impairment of the epithelial barrier.

During 5 minutes of pressure equalization after the basal pressure challenge, tendencies of recovery were observed in some approaches, reflected by an increase in R_T and a decrease in I_{SC}, compared to +5 kPa basolateral applied pressure. However, the approach using 14-day-cultivated cells challenged by a 30-minute-interval did not show a significant recovery, which is inconsistent with the supposed maturing and adaption processes. Nevertheless, the partial reversion of pressure induced effects indicates viability of the cells and the following immunofluorescent staining of tight junction proteins verified that structural integrity of the monolayer was undisturbed, though. Therefore, it may be assumed that increasing basal hydrostatic pressure was applied in a tolerable amount to HC11 monolayers and successfully induced protective reactions of the epithelial barrier – in dependence of maturing status and incubation time.

Differentiation of HC11 monolayers, induced by hormone supplementation with prolactin and dexamethasone, resulted in a significant higher R_T after 14 days of cultivation, which has been described as consequence of tight junction formation in HC11 cells before (Stelwagen *et al.* 1999). However, in the 4-hour-incubation with bilateral 10 kPa hydrostatic pressure, R_T showed no marked changes in all experimental series. This outcome stands in contrast to the results obtained from basal pressure incubations, and preliminary studies performed with apical pressure of 5 kPa in a 4-hour-incubation, which both resulted in a strong decrease of R_T. Bilateral hydrostatic pressure most likely simulates a situation, when mammary epithelial cells are challenged by an increase in pressure due to milk accumulation from apical, and simultaneously are pressurized with back pressure created by the interstitium from the basal side. That the 4-hour-incubation of bilateral pressure did not induce a significant reduction of R_T is in accordance with the finding, that tight junctions become leaky after 18 and 21 hours in cows and goats, respectively (Stelwagen *et al.* 1997, Stelwagen *et al.* 1994). Moreover, in differentiated cells a decrease of I_{SC} was observed during bilateral pressure incubation. This change may have been induced by a sealing reaction of the tight junction due to increased hydrostatic pressure.

Pressure-induced interactions of barrier and transport mechanisms

On the molecular level, a sealing of the tight junction most likely is reflected by upregulation of tightening tight junction proteins, as observed for claudin-1 and -3 after 20 hours of milk cessation in mice (Markov *et al.* 2012). In the present study, 4 hours of hydrostatic pressure incubation did not affect claudin-3 and -4, which nevertheless were continuously expressed in the tight junction of HC11 monolayers after pressure incubation, as confirmed by immunofluorescence microscopy. Whereas claudin-4 was also unaffected as well *in vivo* (Markov *et al.* 2012), an increase of claudin-3 was not observed after hydrostatic pressure

incubation. This discrepancy may have different possible reasons: It may indicate (I) that pressure might not be a regulative factor for claudin-3, (II) that an incubation longer than 4 hours would be necessary to induce an upregulation of claudin-3 by pressure, or (III) that pressure affects mammary epithelial tight junction indirectly, and therefore other cells or substances, as *e.g.* hormones would be required to induce claudin regulation. Nevertheless, since claudin-3 and -4 were not affected in the conducted experiments, an independence of pressure changes and claudin expression pattern in the mammary gland can be assumed for these proteins. Moreover, it implicates that pressure may not be an influencing factor regarding other known functions of claudin-3 and -4, *e.g.* their different described roles in mammary gland neoplasia (Jakab *et al.* 2008, Ma *et al.* 2015, Todd *et al.* 2015).

Throughout their investigations on claudin compositions during the phases of lactation in mice, Kobayashi and Kumura found a higher molecular weight form of claudin-3. The appearance of this form around parturition suggests, that a phosphorylation of claudin-3 may be necessary in the sealing of the tight junction (Baumgartner *et al.* 2017, Kobayashi and Kumura 2011). However, the presence of claudin-4 in the tight junction of differentiated HC11 cells, which normally is absent after the first few days of lactation in mice, together with the fact that no higher molecular weight form of claudin-3 was detected in these monolayers, implicates the expression of an early lactation state in HC11 monolayers. These observations are in accordance with the differentiation protocol, as hormone induction with prolactin and dexamethasone was performed for three days. However, since HC11 monolayers showed a significant higher R_T after hormone induction compared to undifferentiated monolayers, a sealing process of the tight junction can be assumed. To elucidate the role of a higher molecular weight form of claudin-3 in this process, and to further determine the function of claudin-3 and -4 in mammary gland epithelium, is a prerequisite for a detailed understanding of the sealing process and the barrier function during pressure changes in the mammary gland. Therefore, it would be interesting to further evaluate if other claudins, *e.g.* claudin-1, are affected by pressure. Baumgartner *et al.* conclude that claudin-1, -3, -4, -7 and -8 should be considered the most important claudins in the mammary gland (Baumgartner *et al.* 2017). Consequently, it might be promising to investigate if claudin-1, -7 or -8 are affected by hydrostatic pressure.

Since claudin expression and R_T were not changed by bilateral hydrostatic pressure, it was hypothesized that the observed decrease of I_{SC} in differentiated cells during the 4-hour-incubation was originated in ion transport mechanisms. Mechanical stimuli for ion secretion are described in various organs: *e.g.* an activation of chloride conductance induced by apical hydrostatic pressure in lung tissue (Bogdan *et al.* 2008), but also including intestinal tissue (Kaczorowski *et al.* 2010), the gall bladder (Eldrup *et al.* 1982) and the urinary bladder

(Ferguson *et al.* 1997, Wang *et al.* 2003). Employing barium chloride as blocking substance, chloride secretion was shown to be likely responsible for the decrease of I_{SC} after two hours of bilateral pressurization in differentiated cells. Chloride secretion can be blocked by barium chloride *via* inhibition of inward rectifier potassium channels. This inhibits the potassium recirculation and thereby a prerequisite for chloride uptake into the cell *via* NKCC1, a sodium-potassium-chloride-cotransporter (Kreusel *et al.* 1991, Silva *et al.* 1986). An expression of NKCC1 was reported for both, lactating mammary epithelial cells (Shillingford *et al.* 2002) and HC11 cells (Selvaraj *et al.* 2000). During bilateral pressure incubation, barium chloride-induced blockage of chloride secretion was observed after 2, but not after 4 hours of incubation, indicating a proceeding of adaptive processes in hormone induced HC11 monolayers. Additionally, in undifferentiated cells, a high variability of I_{SC} was observed with addition of barium chloride. Both, possible adaptive mechanisms and the variability of I_{SC} could be approached by the implementation of another inhibitor. However, the high amounts of experimental solution necessary for an incubation with 10 kPa hydrostatic pressure in the modified Ussing chamber are a limitation for the use of inhibitors. Nevertheless, it could be beneficial to employ *e.g.* the NKCC1-blocker bumetanide (Kreusel *et al.* 1991) in future studies.

On the molecular level, ion transport induced by mechanical stimuli can be attributed to the activity of membrane mechanosensitive ion channels (Hamill and Martinac 2001, Sachs 1992). It is known that these channels can respond to stimuli of different origin and nature and are capable of distinct reactions that vary in channel-specifying parameters like selectivity and conductivity. Examples may be different channels responsive to membrane tension and changes in membrane curvature, which were described in astrocytes (Bowman *et al.* 1992). Stretch-activated and stretch-inactivated potassium channels were reported to coexist in neurons (Morris and Sigurdson 1989), and pressure-induced stretching of lung tissue resulted in a mechanosensitive activation of CFTR (Vitzthum *et al.* 2015). Since the chloride channel CFTR has also been reported to be expressed in HC11 cells (Selvaraj *et al.* 2000), it can be hypothesized that the observed chloride dependent decrease of I_{SC} during bilateral pressure incubation in HC11 monolayers may be also mediated by mechanosensitive activation of CFTR. However, this theory remains subject to further research. It has nevertheless been demonstrated before that cultured mammary epithelial cells can be stimulated by mechanical forces (Enomoto *et al.* 1987; Furuya *et al.* 1993). This supports the assumption of a pressure induced regulation of barrier functions in mammary epithelial cells.

Dependent on the presence of barium chloride, and therefore related to bilateral pressure-dependent changes of I_{SC} in HC11 monolayers, effects on the expression level of ZO-1 were observed in hormone induced cells. In undifferentiated cells, occludin was affected and not ZO-1, but no effect on I_{SC} was observed in these cells either. In differentiated cells however, ZO-1 was upregulated after 4 hours of pressure incubation, which on electrophysiological level was accompanied by a decrease of I_{SC}. Whereas with addition of barium chloride, a significant decrease of I_{SC} was effectively delayed, and molecular analyses showed a downregulation of ZO-1. Since ZO-1 provides a link between tight junction proteins and the cytoskeleton (Fanning *et al.* 1998; Itoh *et al.* 1999), an I_{SC}-dependent regulation of this protein may reflect an interaction of transport and barrier functions. Synergistic mechanisms of transport and barrier function were reported before, as *e.g.* for sodium absorption in the intestine, which was paralleled by an increase in claudin-8 – preventing a back-leak of sodium through the intercellular space (Amasheh *et al.* 2009, Markov *et al.* 2017). Furthermore, it has been reported that ZO-1 interacts with CFTR *via* a CFTR-PDZ-binding domain and moreover, inhibition of CFTR activity resulted in a reduced expression of ZO-1 (Ruan et a. 2014). Consequently, these findings suggest that a pressure-induced, mechanosensitive upregulation of ZO-1 in HC11 monolayers may have been changed to a downregulation after inhibition of CFTR-mediated chloride transport, due to blockage of inward rectifier potassium channels with barium chloride.

Conclusion

Presented in this thesis, the results obtained from pressure studies revealed adaptive processes in mammary epithelial cells, which involve different aspects of epithelial cell function, including barrier properties, transcellular transport mechanisms and tight junction composition.

As first success of the work a modified Ussing chamber setup was established, which is suitable to apply basal, apical and bilateral hydrostatic pressure in a range of 1 to 10 kPa during electrophysiological measurements, thereby providing stable conditions concerning pH and buffer flow characteristics. This refined technique represents a new tool for the experimental approach of electrophysiological pressure studies, *in vitro*.

Strong changes of electrophysiological parameters were observed during simulation of relatively negative pressure in the mammary gland by application of increasing basal pressure on HC11 monolayers. A partial recovery of electrophysiological parameters after pressurization confirmed the viability of the cells, and the integrity of the epithelial monolayer was verified by immunofluorescent staining of tight junction proteins. Taken together, these findings indicate that the observed changes reflect protective mechanisms of the barrier function, which appear to be of physiological importance during suckling or mechanical milking.

The results presented for bilateral pressure incubation revealed a decrease of I_{SC} in hormone induced HC11 monolayers, which in part was determined by chloride transport mechanisms. In parallel, effects on ZO-1 were observed in dependence of chloride transport. The data suggest an interaction of transport mechanisms and tight junction composition in mammary epithelial cells, which may be mediated by mechanosensitive transduction. These findings highlight the importance of pressure-regulated barrier function under physiological and milk stasis conditions during the lactation period.

Chapter 7: Summary / Zusammenfassung

Analysis of hydrostatic pressure effects on the barrier properties of mammary epithelial cells

During lactation, the cycle of milk synthesis and removal by suckling or milking causes high hydrostatic pressure differences in the mammary gland. To prevent an uncontrolled exchange of milk, blood and intestinal fluid, the mammary gland tissue maintains the blood-milk barrier, which on paracellular level is controlled by the tight junction. In previous studies, Markov *et al.* (2012) found an upregulation of tightening and a downregulation of permeability-mediating tight junction proteins after cessation of milk removal in mice.

The present thesis investigated if and how hydrostatic pressure can mechanically affect the mammary epithelial barrier function. With respect to *in vivo*-approaches as performed by Markov *et al.* (2012), the present thesis further aimed to analyze pressure induced effects isolated from the influence of milk compounds, *in vitro*.

To accomplish this, a method was successfully established to analyze barrier properties during dynamical changes of hydrostatic pressure. For functional analyses of epithelial barrier properties, the Ussing chamber has been proved to be a powerful experimental technique. Therefore, an Ussing chamber was modified with an additional tube-system to apply hydrostatic pressure to the apical, basal or both sides of an epithelial monolayer, in a range of 1 to 10 kPa. The chamber was provided with a manometer and a syringe to dynamically change the pressure during experiments. Employing the maximal pressure level, stable experimental conditions regarding pH and buffer distribution were confirmed.

In the first part of this thesis, effects of negative pressure on the mammary epithelial barrier function were investigated, simulated by increasing basal pressure to monolayers of the mammary epithelial cell line HC11. A combination of different cultivation times (7 and 14 days) and incubation steps (5 and 30 minutes) was employed to further investigate maturing processes and time-dependent adaptation. The basal pressure increase resulted in an immediate and strong decrease of the transepithelial electrical resistance (R_T) in all approaches. In parallel, a successive increase of the short circuit current (I_{SC}) was observed during five steps of +1 kPa basal hydrostatic pressure application. Furthermore, a partial recovery was shown in a following equalization step, and the epithelial monolayer integrity was confirmed *via* immunohistochemical staining of the tight junction. Although the molecular origin remains to be clarified, the obtained data strongly suggest that negative pressure induces protective mechanisms in the mammary epithelial barrier function.

To further elucidate possible adaptive mechanisms of the epithelial barrier during milk stasis within lactation, the second part of the thesis focused on bilateral pressure incubation of HC11 monolayers. 14 day-cultivated HC11 cells were incubated with 10 kPa bilateral hydrostatic pressure for 4 hours and compared with hormone induced HC11 cells in a parallel approach, differentiated by treatment with prolactin and dexamethasone. Whereas no significant changes of R_T were observed, a decrease of I_{SC} was shown in the differentiated cells, which could partly be inhibited by a barium chloride-induced blockage of inward rectifier potassium channels. Moreover, molecular analyses revealed an upregulation of the scaffolding protein ZO-1 in differentiated cells, which was downregulated after an inhibition with barium chloride. Taken together, it can be assumed by the obtained data that a pressure induced chloride transport possibly was mediated by mechanosensitive transduction, while changes of ZO-1 expression might indicate an interaction of transport and barrier mechanisms.

In conclusion, dynamical pressure studies on the mammary epithelial barrier function became assessable by the modified Ussing chamber, which was established as part of this thesis. Moreover, the method may be employed on other cell monolayers and epithelial tissues in future experiments, representing a new tool to study pressure effects, *in vitro*. From the results acquired in both parts of this thesis, it can be conducted that mammary epithelium reacts to hydrostatic pressure with functional and adaptive processes in barrier function, involving tight junction protein composition and transcellular transport mechanisms. These findings elucidate the physiological processes in mammary barrier function and underline the importance to maintain the blood-milk barrier during pressure conditions induced by suckling, milking and times of milk accumulation in the mammary gland.

Zusammenfassung der Dissertation:

Eine Analyse der Effekte hydrostatischen Drucks auf die Barriereeigenschaften epithelialer Milchdrüsenzellen

Während der Laktation werden durch die Abfolge von Milchsynthese und Milchentnahme hohe Druckunterschiede in der Milchdrüse erzeugt. Um einen unkontrollierten Austausch von Milch, Blut und Gewebsflüssigkeit zu verhindern, bildet das Milchdrüsengewebe die Blut-Milch-Schranke aus, welche auf parazellulärer Ebene von der Tight Junction kontrolliert wird. In vorausgegangenen Untersuchungen fanden Markov *et al.* (2012) eine Hochregulierung abdichtender und einer Runterregulierung Permeabilität-vermittelnder Tight Junction-Proteine nach einer Unterbrechung der Milchentnahme bei Mäusen.

Die vorliegende Arbeit untersucht, ob und wie hydrostatischer Druck die Barriereeigenschaften von Milchdrüsenepithel mechanisch beeinflussen kann. Mit Bezug zu den von Markov *et al.* (2012) durchgeführten *in vivo*-Untersuchungen, hatte die vorliegende Arbeit darüber hinaus zum Ziel druckinduzierte Effekte isoliert vom Einfluss der Milchinhaltsstoffe *in vitro* zu analysieren.

Zu diesem Zweck wurde erfolgreich eine Methode etabliert, mit der Barriereeigenschaften während dynamischer Änderungen von hydrostatischem Druck analysiert werden können. Für die funktionale Analyse epithelialer Barriereeigenschaften hat sich die Ussing-Kammer als hervorragende experimentelle Technik erwiesen. Aufgrund dessen wurde eine Ussing-Kammer modifiziert, so dass damit hydrostatischer Druck zwischen 1 und 10 kPa auf die apikale, basale oder beide Seiten eines einschichtigen Epithels gegeben werden kann. Die Kammer wurde mit einem Manometer und einer Spritze versehen, um den Druck während der Experimente dynamisch anpassen zu können. Stabile experimentelle Bedingungen wurden sichergestellt, indem pH und Pufferverteilung unter maximalen Druckbedingungen getestet wurden.

Im ersten Teil der Arbeit wurden die Effekte von Unterdruck auf die Barrierefunktion von Milchdrüsenepithel untersucht, simuliert durch ansteigenden Druck von basal auf konfluenten Zellrasen der Milchdrüsenepithelzelllinie HC11. Eine Kombination aus verschiedenen Kultivierungszeiten (7 und 14 Tage) sowie Inkubationsintervallen (5 und 30 Minuten) wurde verwendet, um Reifungsprozesse und eine zeitabhängige Anpassung näher zu untersuchen. Basaler Druckanstieg resultierte in einem sofortigen und starken Abfall des transepithelialen Widerstandes (R_T) in allen Versuchsreihen. Parallel dazu konnte ein sukzessiver Anstieg des Stroms (I_{SC}) als Reaktion auf fünf Zugaben von je +1 kPa basalem hydrostatischem Druck aufgezeigt werden. Zusätzlich wurde während eines anschließenden Druckausgleichs eine anteilige Erholung beobachtet und die epitheliale Integrität mittels immunhistochemischer Färbung der Tight Junction verifiziert. Obwohl der molekulare Ursprung noch geklärt werden

muss, legen die erhobenen Daten den Schluss nahe, dass Unterdruck im Milchdrüsenepithel protektive Mechanismen der Barrierefunktion hervorruft.

Mit dem Ziel mögliche adaptive Mechanismen der epithelialen Barriere während einer Milchstasis in der Laktation näher zu beleuchten, lag der Fokus des zweiten Teils dieser Arbeit auf beidseitiger Druckinkubation von konfluenten HC11. 14 Tage-kultivierte HC11-Zellen wurden mit 10 kPa beidseitigem hydrostatischem Druck für vier Stunden inkubiert und in einem zweiten Ansatz mit Hormon-induzierten HC11-Zellen verglichen, die durch Behandlung mit Prolaktin und Dexamethason differenziert worden waren. Es wurden keine signifikanten Änderungen des R_T beobachtet, aber es wurde eine Abnahme des I_{SC} in differenzierten Zellen gezeigt, welche teilweise durch Bariumchlorid induzierten Block von einwärtsrektifizierenden Kaliumkanälen inhibiert werden konnte. Darüber hinaus wurde in differenzierten Zellen eine Hochregulierung des Gerüstproteins ZO-1 gezeigt, welches nach Bariumchloridzugabe herunterreguliert war. Zusammengefasst unterstützen die erhobenen Daten die Annahme, dass ein Druck verursachter Chloridtransport wahrscheinlich durch Mechanotransduktion vermittelt wurde, während die Änderungen in der Expression von ZO-1 auf eine Interaktion zwischen Transport- und Barrieremechanismen hindeuten.

Abschließend kann gesagt werden, dass mit der modifizierten Ussing-Kammer, die als Teil dieser Arbeit etabliert wurde, dynamische Druckstudien an der Barrierefunktion des Milchdrüsenepithels möglich gemacht wurden. Darüber hinaus kann die Methode in zukünftigen Experimenten auch mit anderen Zellrasen und epithelialen Geweben eingesetzt werden, und stellt damit ein neues Werkzeug in dar, um Druckeffekte *in vitro* zu untersuchen. Aus den Ergebnissen der beiden Teile dieser Arbeit kann geschlussfolgert werden, dass Milchdrüsenepithel auf hydrostatischen Druck mit funktionellen und adaptiven Prozessen der Barrierefunktion reagiert, einschließlich Änderungen des Proteinexpressionsmusters der Tight Junction und zellulärer Transportmechanismen. Die Ergebnisse beleuchten somit die physiologischen Prozesse der Barrierefunktion in der Milchdrüse und unterstreichen die Relevanz der Aufrechterhaltung der Blut-Milch-Schranke, um Druckänderungen durch Säugen, Melken und Milchstau zu begegnen.

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List of publications

Publications (Peer reviewed)

2018

Mießler KS, Markov AG, Amasheh S

Hydrostatic pressure incubation affects barrier properties of mammary epithelial cell monolayers, *in vitro*

Biochem Biophys Res Commun **495**:1089-1093

Mießler KS, Vitzthum C, Markov AG, Amasheh S

Basolateral pressure challenges mammary epithelial cell monolayer integrity, *in vitro*

Cytotechnology **70**:567-576

Abstracts in proceeding & participation in conferences

2018

Mießler KS, Markov AG, Amasheh S

Effects of bilateral hydrostatic pressure incubation on the barrier function of mammary epithelial cells

Tagung der DVG-Fachgruppe “Physiologie und Biochemie", Wien – 21.02.-23.02.2018

2017

Mießler KS, Vitzthum C, Markov AG, Amasheh S

Effects of basal hydrostatic pressure on differentiated and undifferentiated mammary epithelial cells

96th Annual Meeting of the German Physiological Society, Greifswald – 16.-18.03.2017

Acta Physiologica; 219, Supplement 711, S. 90

2016

Mießler KS, Amasheh S

Establishing a modified Ussing chamber to analyze effects of hydrostatic pressure on epithelial cells *in vitro*

5. Symposium der Jungen Physiologen, Jülich – 22.09.-23.09.2016

Mießler KS, Vitzthum C, Markov AG, Amasheh S

Effects of hydrostatic pressure on barrier function of epithelial cells

International conference: Tight junctions and their proteins, Berlin – 08.-10.09.2016

Mießler KS, Markov AG, Vitzthum C, Amasheh S

Effects of hydrostatic pressure on epithelial cells: the barrier function of the mammary gland

Tagung der DVG-Fachgruppe “Physiologie und Biochemie", Berlin – 30.03.-01.04.2016

Mießler KS, Vitzthum C, Markov AG, Amasheh S

Effects of hydrostatic pressure on epithelial cells: approaches to analysis of mechanical force in a mammary gland model, *in vitro*

70. Jahrestagung der Gesellschaft für Ernährungsphysiologie, Hannover – 08.-10.03.2016

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Mießler KS, Markov AG, Amasheh S

Effects of hydrostatic pressure on epithelial cells: establishing a modified Ussing chamber technique

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2015

Markov AG, Mießler KS, Amasheh S

Dynamic regulation of tight junction protein localization in murine mammary gland epithelium

69. Jahrestagung der Gesellschaft für Ernährungsphysiologie, Göttingen – 10.-12.03.2015

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Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Potsdam, am Unterschrift

Author Contribution

(1) Mießler KS, Markov AG, Amasheh S (2018) Hydrostatic pressure incubation affects barrier properties of mammary epithelial cell monolayers, *in vitro*. Biochem Biophys Res Commun 495:1089-1093

I have designed and conducted experiments, including immunoblotting, immunofluorescent staining and data analysis. I wrote the article.

(2) Mießler KS, Vitzthum C, Markov AG, Amasheh S (2018) Basolateral pressure challenges mammary epithelial cell monolayer integrity, *in vitro*. Cytotechnology 70:567-576

I have designed and conducted experiments, including establishment of the method. Electrophysiology and immunofluorescence data were acquired by me personally, and I have performed data analysis and discussion.

Potsdam, the Signature

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