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TRANSPORT PHYSIOLOGY

Altered expression of tight junction proteins in mammary epithelium after discontinued suckling in mice

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Abstract Milk production is modulated by the paracellular barrier function of tight junction (TJ) proteins located in the mammary epithelium. The aim of our study was the molecular analysis of TJs in native lactating murine mammary gland epithelium as this process may strongly challenge epithelial barrier properties and regulation. Mammary gland tissue specimens from lactating control mice and animals after a 20-h interruption of suckling were prepared; histological analyses were performed by light and electron microscopy; and expression of TJ proteins was detected by PCR, Western blotting, immunofluorescent staining, and confocal laser scanning microscopy. Discontinuation of suckling resulted in a substantial accumulation of milk in mammary glands, an increase of alveolar size, and a flattening of epithelial cells without effects on inflammatory indicators. In control tissues, PCR and Western blots showed signals for occludin, and claudin-1, -2, -3, -4, -5, -7, -8, -15, and -16. After a 20-h accumulation of milk, expression of two sealing TJ proteins, claudin-1 and -3, was markedly increased, whereas two TJ proteins involved in cation transport, claudin-2 and -16, were reduced. Real-time PCR validated increased transcripts of claudin-1 and claudin-3. During extension of mammary glands in the process of lactation, claudin-1 and -3 are markedly induced and claudin-2 and -16 are decreased. Volume and composition of milk might be strongly dependent on this counter-regulation of sealing claudins with permeability-mediating claudins, indicating a physiological process of a tightening of TJs against a back-leak of solutes and ions from the alveolar lumen.

Keywords Mammary gland · Lactation · Claudins · Occluding

Abbreviations
TJ Tight junction

Introduction

Mammary gland alveolar epithelium consists of cuboidal cells which are responsible for milk synthesis and secretion. Prerequisite for vectorial transport through epithelia is a paracellular barrier function which is provided by the tight junction (TJ) [6, 41]. TJs have been identified in the apicolateral region of alveolar cuboidal cells and are involved in the selectivity and regulation of the passage of solutes through the paracellular pathway, maintaining secretion formation in alveoli [32].

TJs are organized in strands, and within these strands, several tetraspan TJ proteins have been reported to contribute to barrier function, namely occludin [16], tricellulin [22, 26], marvel D3 [38], and the family of claudins [14]. Among these TJ proteins, the claudin family has been demonstrated to primarily determine barrier properties but, in contrast, also selective paracellular permeability to ions, larger solutes, and water in a wide

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variety of epithelia, especially in the two tubular epithelia of kidney and intestine (for review, see [4]). Some of them, as claudin-1, -3, -5, and -8, markedly seal the TJ [6, 7, 15, 30]. In contrast, other claudins have been demonstrated to specifically mediate paracellular permeability as it has been shown in detail for claudin-2 which forms a paracellular cation and water channel [5, 36], and claudin-16, which influences epithelial permeability for Mg^{2+} and Ca^{2+} [24].

Expression of claudin-1, -3, -4, -5, -7, -8, -15, and -16 has been detected in mammary gland cell lines [19, 35], and some of these proteins have been detected in mammary gland tissue, including claudin-1, -2, -3, -4, -5, and -7 [9, 10, 23]. These studies highlight aspects of claudin expression, e.g., as tumor or developmental markers, but a specific correlation with the physiological process of lactation has not been elucidated in detail, so far. An explanation may be that, although in vitro mammary cell models are available, factors like intramammary pressure are not present, but may be required in the regulation of mammary tight junctions [12, 33]. Taking into consideration that milk secretion may challenge alveolar barrier integrity, analysis of the effect of milk accumulation in alveoli was performed to analyze the contribution and regulation of tight junctions to the physiological process of lactation.

Materials and methods

Animals and mammary gland preparation

A standard laboratory mouse strain, C57Bl/6 J, was obtained from the Russian Academy of Sciences, St. Petersburg, Russia, and experiments were performed at the period of *steady-state* lactation on the 10–15th day after delivery. Animals obtained standard food and water ad libitum. In the experimental approach, pups were separated from female mice 20 h before tissue preparation. Animals were sacrificed by CO_2 asphyxiation. In the control group, glands were taken without an interruption of weaning. Mammary gland preparations were weighed, and tissue samples were prepared for histological analyses by light and electron microscopy, for Western blotting, and for confocal laser scanning immunofluorescence microscopy. Experimental procedures were in conformity with regulations of the Local Ethics Committee of St. Petersburg State University, Russia, which are in accordance with the European convention for the protection of vertebrate animals used for scientific purposes.

Histological analysis and detection of inflammatory markers

Histological analysis was performed as reported recently [6]. Briefly, tissues were fixed in 3% formalin for 1 h. After a standard protocol employing increasing ethanol concentrations, samples were paraffined. The mounts were arranged on sample glasses, deparaffined, and toluidine-stained sections were obtained and analyzed by optical microscopy.

Morphometrical characteristics were analyzed in cross sections of mammary glands of controls and experimental groups. As alveoli have an elliptical shape, therefore the diameters of the length and width were analyzed. Furthermore, the height of cuboidal epithelial cells was analyzed.

For detection of inflammatory markers, terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining was employed to detect apoptoses, and hematoxylin and eosin (H&E) staining was performed for the detection of lymphocyte infiltration, as reported previously [40]. Briefly, paraffin-fixed samples were cut into 4-μm-thick sections, deparaffined and stained with H&E for detection of lymphocytes, or, for detection of epithelial apoptoses, with TUNEL assay kit (Roche, Mannheim, Germany), respectively. Subsequently, the number of positive intraepithelial lymphocytes and the rate of epithelial apoptosis were determined per 1,000 epithelial cells, respectively.

Electron microscopy

Tissue samples were fixed by immersion fixation in 2.5% glutaraldehyde and Hanks' balanced salt solution, pH=7.0, at 4°C for 2 h. After washing in Hanks' solution, the tissue was postfixed in osmium tetroxide solution (1% OsO_4 in Hanks' solution) at 4°C for 2 h, block-stained in 2% uranyl acetate buffer at 40°C for 1 h, dehydrated in ethanol and acetone, and embedded in Spurr. Thin sections were obtained on the ultramicrotome LKB-8800 (LKB, Sweden) and were stained in uranyl acetate and lead citrate. The sections were examined employing a JEM-100C microscope (JEOL, Japan).

Immunoblots

Immunoblots were performed as described in detail previously [6, 8, 13]. Tissues and cells were homogenized in Tris buffer containing 20 mM Tris, 5 mM $MgCl_2$, 1 mM EDTA, 0.3 mM EGTA, and protease inhibitors (Complete, Boehringer, Mannheim, Germany), and subsequent passage through a 26 G 1/2-in. needle. Membrane fractions were obtained by two centrifugation steps (5 min at 200×*g* and 30 min at 43,000×*g*, 4°C). Pellets were resuspended in Tris buffer. Protein contents were determined using BCA Protein assay reagent (Pierce, Rockford, IL, USA) and quantified with a plate reader (Tecan, Grodig, Austria). Samples were mixed with SDS buffer (Laemmli), loaded on a 12.5% SDS polyacrylamide gel, and electrophoresed.

Proteins were detected by immunoblotting employing primary antibodies raised against occludin and claudins

used in concentrations of 1:1,000 and 1:5,000 according to the manufacturer's protocols (Invitrogen, San Francisco, CA, USA), and beta-actin (Sigma, Taufkirchen, Germany). Peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG antibodies and the chemiluminescence detection system Lumi-LightPLUS Western blotting kit (Roche, Mannheim, Germany) were used to detect bound antibodies. Signals were visualized by luminescence imaging (LAS-1000, Fujifilm, Tokyo, Japan). For comparison of Western blot signals, densitometry was performed employing AIDA Raytest 2.5 software (Straubenhardt, Germany).

Immunostaining and confocal laser scanning microscopy

Immunostaining of tissues was performed as described in detail recently [3, 29]. Tissues were fixed in formalin for 2 h at 4°C and embedded in paraffin. For immunostaining, paraffin was removed from cross sections (8 μm) by a xylol–ethanol gradient. For antigen retrieval, sections were boiled in 1 mM EDTA or 10 mM citrate buffer solution. To block non-specific binding sites, tissues were bathed in PBS containing 5% (vol vol^{-1}) goat serum (blocking solution) and 1% BSA for 60 min at room temperature. All subsequent washing procedures were performed with this blocking solution. For immunostaining, monoclonal mouse anti-occludin antibody was employed (Invitrogen), diluted 1:200 in blocking solution. Tissues were incubated for 60 min and, after two washes, were incubated with Alexa Fluor goat anti-rabbit IgG for 45 min (Molecular Probes, USA, diluted 1:500 in blocking solution). Sections were mounted with ProTags MountFluor (Biocyc, Luckenwalde, Germany). Fluorescence images were obtained with a confocal laser scanning microscope (LSM 510 Meta, Zeiss, Jena, Germany).

PCR experiments

Preparation of mRNA and quantitative PCR employing pre-designed TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) was performed as reported in detail previously [6]. Copy numbers were normalized to GAPDH copies. TaqMan primers were FAM- (claudins) or VIC-labeled (GAPDH). No template controls (NTC) were employed as negative controls. Real-time quantitative reverse transcription–PCR was performed with a 7500/7900HT Fast Real-Time PCR System in conjunction with respective software (SDS2.2.2; Applied Biosystems).

Statistical analysis

Data are expressed as means ± SEM. Statistical analysis was performed using Student's *t* test. $P < 0.05$ was considered significant.

Results

Histological analysis of mammary gland alveoli

Morphology of mammary gland alveoli was analyzed in toluidine-stained sections of formalin-fixed tissue samples (Fig. 1a). Mammary lobules were separated by a thin interlayer of connective tissue that contained blood vessels. There was little adipose tissue. Alveolar lumina were lined by a single layer of epithelial cells, the cuboidal cells. After a 20-h interruption of suckling, the milk cumulated in mammary alveoli, with a markedly increased amount of fat drops in the alveolar cavity (Fig. 1b).

After preparation of the mammary glands, weight of single preparations was recorded, showing an about 3-fold increase of weight from 249±35 mg to 727±85 mg (**$p<0.01$, $n=5$ and 6, Fig. 2a). Furthermore, morphometric analysis showed that the cuboidal cell height after a 20-h interruption of suckling was markedly decreased (Fig. 2b). The size of alveoli was determined by width and length measurements (Fig. 2c, d). Both parameters were markedly increased apparently due to storage effects in alveoli after extended interruption of suckling.

Ultrastructural analysis of alveoli by transmission electron microscopy revealed typical features of secretory epithelial cells (Fig. 3). Microvilli were detected in the apical membrane and secretory vesicles were found in the apical parts of the cells. Nuclei and numerous mitochondria were detected in the basal area of secretory epithelial cells. In both sets of gland preparations, controls and tissues after 20-h interruption of suckling, TJs were detected in the apicolateral membrane.

Expression of tight junction proteins

Protein preparations were performed to detect TJ proteins in mammary gland tissues. Western blots revealed specific signals for occludin, and claudin-1, -2, -3, -4, -5, -7, -8, -15, and -16 in gland tissue of controls and after interruption of suckling (Fig. 4a). Densitometric analysis of Western blots

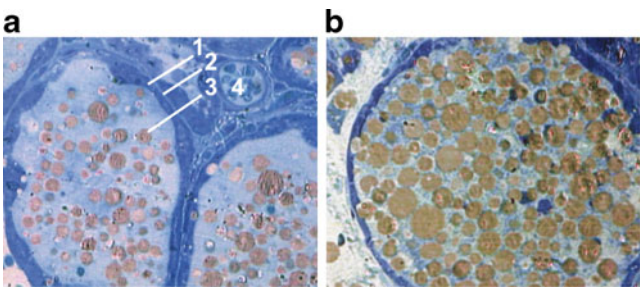


Fig. 1 Histology of mammary gland alveoli (*1* epithelial cell, *2* basal lamina, *3* fat vacuole, *4* blood vessel). *Left*—control tissue. *Right*—alveoli after an interruption of suckling for 20 h: secretion accumulation and flattening of cuboidal epithelial cells (toluidine blue staining, magnification×240)

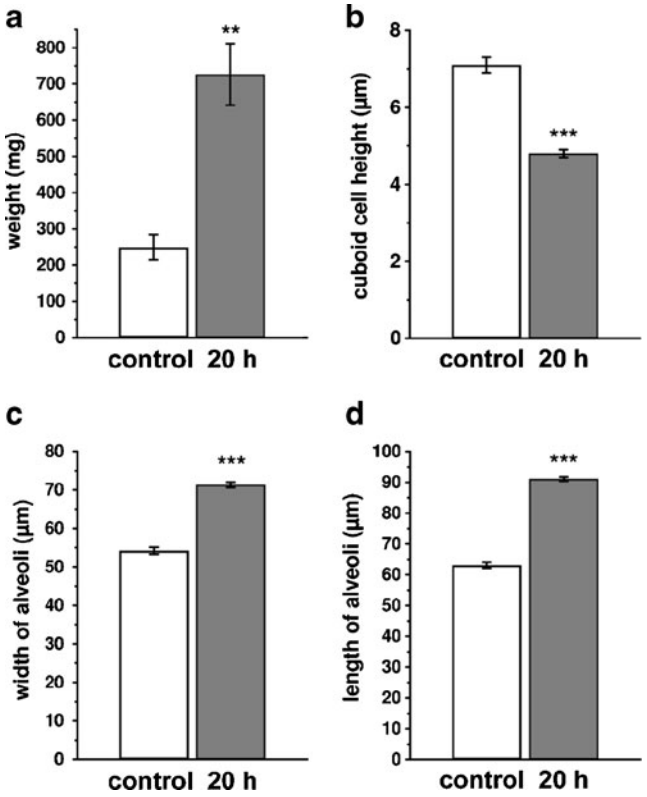


Fig. 2 Parameters of alveoli and epithelial cell morphology. **a** Weight of mammary gland preparations. Preparations of mammary glands showed a 3-fold increase in weight. **b** Cuboidal cell height. A significant flattening of cuboidal cells was observed after an interruption of suckling for 20 h. **c** Width of alveoli. **d** Length of alveoli. Both width and length of alveoli were markedly increased after an interruption of suckling for 20 h (**$p<0.01$, ***$p<0.001$, $n=5$ and 6 animals, respectively)

revealed marked changes of single claudins after the extended period of lactation without suckling (Fig. 4b). Claudin-1 and -3 were markedly increased compared to controls. In contrast, claudin-2, which forms paracellular channels for small cations and water, as well as claudin-16, which indirectly mediates divalent cation permeability, were decreased. Densitometry revealed no significant change of claudin-4, -5, -7, -8, -15, and occludin signals compared to controls, though.

Quantitative PCR of induced sealing claudins

TaqMan PCR revealed a marked increase claudin-1 and -3 mRNA copy numbers in accordance with increased Western blot signals. Quantification of nucleic acids revealed a 16.1±2.7-fold increase of claudin-1 mRNA copy numbers and a 5.0±1.3-fold increase of claudin-3 mRNA copy numbers after 20 h of interrupted suckling, whereas mRNA of GAPDH, which served as internal standard, and mRNA of claudin-8, did not change ($n=5$, **$p<0.01$, and ***$p<0.001$, Fig. 5).

Detection of claudins by confocal laser scanning microscopy

Confocal laser scanning immunofluorescence microscopy revealed strong and consistent signals for occludin in the apicolateral membrane of cuboidal cells in mammary gland epithelium of control tissues (Fig. 6, green staining). Co-stainings revealed a strong co-localization of red signals for the majority of claudins with occludin (Fig. 6, red staining). Merged images revealed consistent results with Western blot experiments: signals of claudin-1 and -3 were increased after the extended lactation period and claudin-16 was decreased compared to controls. However, immunostainings of claudin-2 were rather weak within tight junction complexes already in controls and after 20 h of interrupted suckling compared to respective Western blot signals and compared to detection of major sealing tight junction proteins claudin-1 and -3.

Detection of inflammatory markers

HE stainings were performed to analyze infiltration of lymphocytes. Lymphocytes were, if at all, only marginally detected in both controls and 20 h after suckling (not shown). Moreover, TUNEL staining revealed 2.7±0.7‰ apoptoses in controls, with no significant change after 20-h interruption of suckling (3.0±0.7‰ apoptoses, $n=7$, respectively).

Discussion

Alveoli during regular and interrupted suckling

Mice do not have any sinuses and cisterns required for secretion storage. In our study, morphometric changes of alveoli and epithelial cells during an interruption of suckling indicate a change of hydrostatic pressure inside

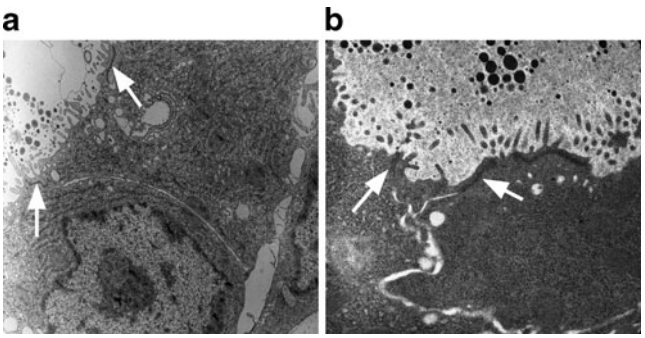
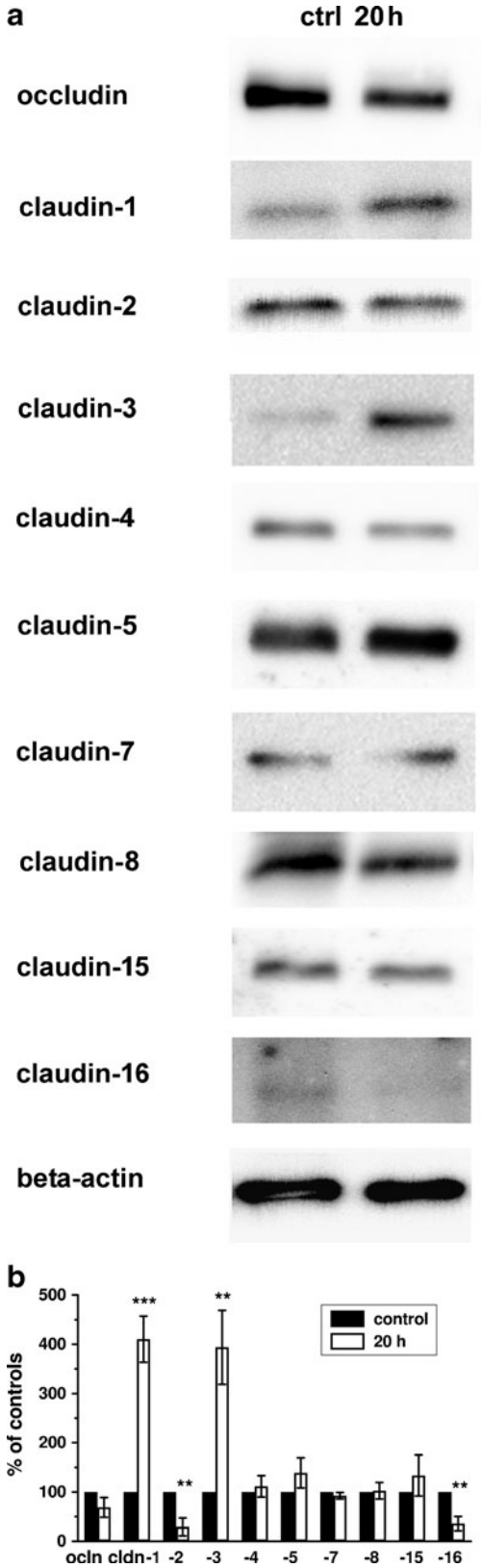


Fig. 3 Electron microscopy of murine cuboidal mammary epithelium cells. **a** Control, **b** experimental approach. In both controls and cells after a 20-h interruption of suckling, tight junctions were detected within the apicolateral membrane of cuboidal epithelial cells, without visible changes of tight junction localization and integrity



◀ **Fig. 4** Detection of tight junction proteins. **a** Western blots, **b** densitometry. Occludin, and claudin-1, -2, -3, -4, -5, -7, -8, -15, and -16 were detectable in control tissues. After a 20-h interruption of suckling, claudin-1 and -3 were increased, whereas claudin-2 and claudin-16 were decreased. No significant changes were detected for claudin-4, -5, -7, -8, -15, and occludin (n=4–7, respectively, $^{**}p<0.01$, $^{***}p<0.001$)

alveoli. Regularly, mice can have intervals between pups suckling of more than 2 h [28]. Independent from this condition, a continuous flux of secretion into the lumen takes place. Constitutive secretion was detected by measurement of labeled amino acids in mammary gland epithelium and its secretion [42]. The secretion storage and the increase of hydrostatic pressure occur initially in the cavity of alveoli. Analysis of mammary glands after discontinued suckling is a "classic" approach for analyses of lactation, which dates back to the 1970s. In 1973, Lincoln et al. focused on an 18-h interval for comparison of pup's weight [27]. In 1986, Higuchi et al. have employed a 15-h time interval for analyses of oxytocin in plasma [18]. During extended periods after suckling, the amplitude of myoepithelial cell contractions significantly increases in response to test application of oxytocin, and the transepithelial voltage reaches a minimum after 20 h [1, 39].

Based on previous observations in mammary gland epithelia, we tested the hypothesis that tight junction proteins might be involved in a sealing mechanism within the alveolar epithelium to antagonize adverse effects of hydrostatic pressure in alveoli, and therefore to support a maintenance of the alveolar epithelium integrity.

The changes indicate a modulation of barrier integrity during lactation, with an increase of barrier-forming tight junction proteins and a decrease of permeability-mediating claudins. These changes functionally indicate a general sealing of the paracellular barrier in mammary gland epithelium during an extended period of lactation without suckling, leading to an accumulation of milk and an increase of hydrostatic forces in mammary gland alveoli.

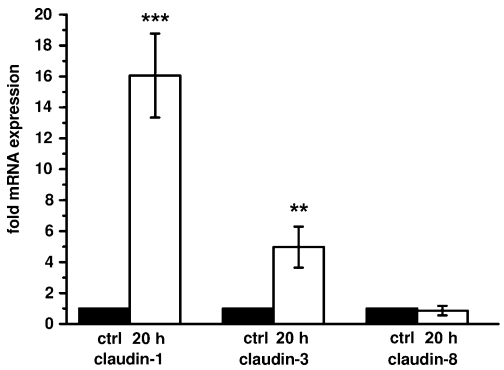


Fig. 5 Transcriptional regulation of major sealing TJ proteins. A marked increase in claudin-1 and -3 mRNA copy numbers was detected by quantitative PCR. In contrast, mRNA transcripts of claudin-8, another sealing TJ protein, was not changed (n=5, respectively, $^{**}p<0.01$, $^{***}p<0.001$)

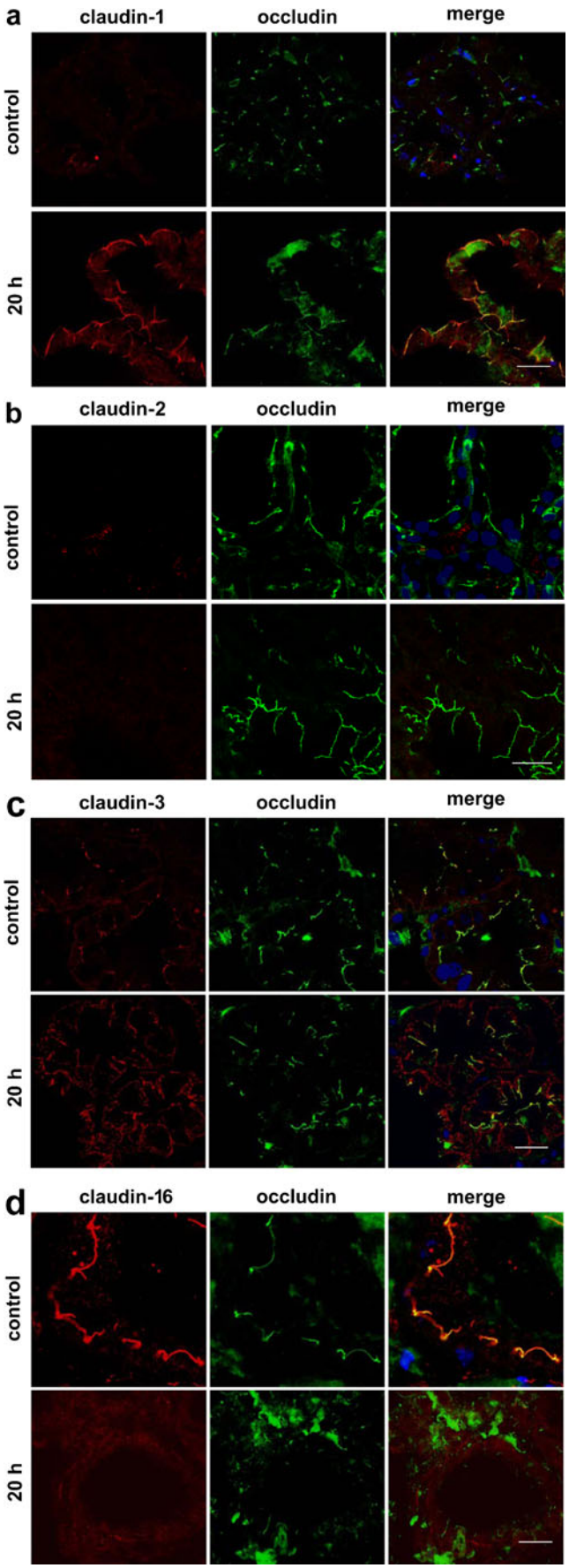


Fig. 6 Detection of tight junction proteins by means of confocal laser scanning immunofluorescence microscopy. Occludin, and **a** claudin-1, **b** claudin-2, **c** claudin-3, and **d** claudin-16. Immunofluorescent staining of claudin-1, claudin-3, and claudin-16 revealed a marked co-localization with occludin within the tight junction complexes of cuboidal cells lining the alveolar lumen. In controls, a strong signal of claudin-16 was detected, whereas claudin-1 signals were weaker. After a 20-h interruption of suckling, claudin-1 and claudin-3 signals were markedly increased, whereas a strong decrease in claudin-16 signals was observed. Detection of claudin-2 was relatively weak in controls, but further decreased after 20-h interruption of suckling (*bar*=20 μm)

Tight junctions as a local regulator of lactation homeostasis

In our study, claudin-1, -2, -3, -4, -5, -7, -8, -15, and -16 have been detected in native murine mammary gland epithelia. Occludin was employed as a general marker of tight junction localization, which has been evaluated in previous studies [5, 7]. Expression of TJ proteins, however, varies between a continuous lactation and an interruption of suckling. The TJ proteins detected in our study have been functionally characterized in detail recently employing stably transfected epithelial cell lines and mouse models. From these studies, the detailed information concerning the contribution of occludin and members of the claudin family to barrier properties emerged.

Mice deficient for claudin-1 die within hours after birth because of dehydration. These animals show a severe weight loss due to evaporation of water through the skin [15]. Therefore, claudin-1 is regarded as one major barrier-building tight junction protein, and induction of this protein within tight junction complexes as reported here is strongly linked to a physiological sealing of TJs.

In contrast, claudin-2 has been demonstrated to form a paracellular channel selective for small cations [5] as well as for water [36]. Therefore, claudin-2 can be regarded as an important mediator of paracellular leakiness which is supported by its strong expression in proximal nephron segments and intestine (for review, see [4]). In our study, however, visualization of claudin-2 within TJs of mammary gland alveoli was limited, although a strong decrease of signals was detected by Western blotting. Even though only scarcely detected within alveoli tight junctions by immunofluorescent staining, this decrease may still markedly contribute to the process of lactation due to its key role as a paracellular channel.

Claudin-3 is ubiquitously expressed in many epithelia, including intestine [29], kidney [25], and endothelia [45]. The functional characterization of claudin-3 revealed a general barrier-forming role, sealing the paracellular pathway against the passage of ions and macromolecules [30]. Increased expression of claudin-3 as reported in this physiological context of mammary gland function can be expected to have a major impact on barrier properties towards a sealing of the TJ.

In contrast, further TJ proteins known to contribute to the sealing of the TJ remained unchanged and therefore can be regarded to contribute to basic TJ formation, as claudin-4, -5, and -8. The sealing effect of these proteins has been demonstrated in transfection studies [7, 44, 46]. Among these proteins, claudin-5 plays a central role as a major endothelial TJ protein [34], and claudin-8 had been reported to be regulated during ENaC induction in the colon [6]. However, the stable expression of these important determinants of barrier function in our current study underlines the differential physiological regulation of tight junctions in different organs.

More ambiguous functions have been attributed to two further claudins presented in this study, namely claudin-7 and -15. Both claudins had been reported to mediate cation permeability in LLC-PK1 cells [2, 43], but have also tightening properties depending on the employed cell model (for review, see [4]).

Finally, a major role for the uptake of Ca^{2+} and Mg^{2+} in kidney epithelia was attributed to claudin-16 [24, 37]. However, claudin-16 alone appears not to act by forming Ca^{2+} and Mg^{2+} channels by itself but regulates divalent cation transport by indirect means [17, 21]. Therefore, a decrease of claudin-16 may contribute to prevent back-leakage of secreted Ca^{2+} and Mg^{2+} from the alveolar lumen into serosal tissue.

Milk Ca^{2+} content is derived quantitatively through exocytosis of secretory vesicles, leading to a several-fold higher Ca^{2+} concentration than in the blood (7.5 mM vs. 2.23 mM [31]). Transepithelial voltage of alveolar epithelium is −30 mV, and after 20-h interruption of suckling this voltage decreases to 0 mV [39]. Thereby a driving force for ion flux disappears, but transepithelial resistance remains high. An explanation for these changes is in accordance with the observed change of tight junction protein expression. Moreover, the change of transepithelial voltage could be a direct effect of a changed cation-to-anion permeability ratio. In our model, these changes could, e.g., be due to changes of claudin-2, which produces a high permeability ratio for cations over anions [5]. However, the observed voltage changes might not be attributed exclusively to one tight junction protein in favor to a more complex co-localization of different TJ proteins, as reported also for kidney and intestine [4]. Regarding claudin-2, a second aspect becomes relevant as studies on claudin-2 have shown that this single claudin markedly determines the paracellular permeability for water [36]. Therefore, a change of claudin-2 might change the hydraulic water permeability in a way that the milk is concentrated by back-filtration of water or water and osmolytes. Possible regulatory stimuli for these changes could be manifold, but may be most likely attributed to a local mechanical stimulus as a marked change of epithelial cell morphology occurs in parallel to milk accumulation. Therefore, it is likely that mechanical stress may change TJ composition, which is also discussed, e.g., for urothelia induced by the filling (and rapid emptying) of the urinary bladder [11] and in wound healing models [20].

In conclusion, our study for the first time provides data on tight junction protein expression in native murine mammary gland epithelium in combination with regulatory mechanisms occurring during lactation. During an interruption of suckling, an increase of barrier-forming TJ proteins and a decrease of permeability-mediating TJ proteins is observed, which well explains the physiological mechanisms involved for homeostasis of milk production and composition during lactation.

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Conflict of interest There is no conflict of interest.

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