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**Prolactin and glucocorticoid signalling induces
lactation-specific tight junctions concurrent with β -casein
expression in mammary epithelial cells**

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ABSTRACT

Alveolar mammary epithelial cells (MECs) in mammary glands are highly specialized cells that produce milk for suckling infants. Alveolar MECs also form less permeable tight junctions (TJs) to prevent the leakage of milk components after parturition. In the formation process of less permeable TJs, MECs show a selective downregulation of Cldn4 and a localization change of Cldn3. To investigate what induces less permeable TJs through these compositional changes in Cldns, we focused on two lactogenesis-related hormones: prolactin (Prl) and glucocorticoids. Prl caused a downregulation of Cldn3 and Cldn4 with the formation of leaky TJs in MECs *in vitro*. Prl-treated MECs also showed low β -casein expression with the activation of STAT5 signalling. In contrast, dexamethasone (Dex), a glucocorticoid analogue, upregulated Cldn3 and Cldn4, concurrent with the formation of less permeable TJs and the activation of glucocorticoid signalling without the expression of β -casein. Cotreatment with Prl and Dex induced the selective downregulation of Cldn4 and the concentration of Cldn3 in the region of TJs concurrent with less permeable TJ formation and high β -casein expression. The inhibition of Prl secretion by bromocriptine in lactating mice induced the upregulation of Cldn3 and Cldn4 concurrent with the downregulation of milk production. These results indicate that the coactivation of Prl and glucocorticoid signalling induces lactation-specific less permeable TJs concurrent with lactogenesis.

1. Introduction

The mammary gland is a highly specialized organ that supplies milk to suckling infants. Milk is produced by alveolar mammary epithelial cells (MECs) in lactating mammary glands. Alveolar MECs proliferate during late pregnancy and develop the alveolar structure. However, alveolar MECs do not initially have the ability to supply enough milk during pregnancy. Substantial milk production by alveolar MECs is initiated by stimulation from prolactin and glucocorticoids around the time of parturition [1, 2]. Prolactin activates the signal transducer and activator of transcription 5 (STAT5)/Janus kinase 2 (JAK2) pathway to induce the transcription of specific sets of lactogenesis-related genes in MECs [3, 4]. Glucocorticoids bind to the intracellular glucocorticoid receptor (GR) to form the glucocorticoid/GR complex, which acts as a transcriptional coactivator of Stat5 to enhance Stat5-dependent transcription of lactogenesis [5-8]. Therefore, coactivation of both the Prl/JAK2 and glucocorticoid/GR pathways is indispensable for substantial milk production in alveolar MECs during lactation.

Lactating alveolar MECs have less permeable tight junctions (TJs) to prevent the leakage of milk components and body fluids across the alveolar lumen to the mammary interstitium during lactation [9-11]. However, non-lactating alveolar MECs have permeable TJs that allow the leakage of milk components and body fluids [12, 13]. The less permeable TJs are

established shortly after parturition and are maintained throughout lactation [11, 14]. Linzell and Peaker suggested that the closure of TJs after parturition is necessary for the compositional changes that occur as colostrum is converted to mature milk [15]. In lactating mammary glands, a temporal disruption of mammary TJs by treatment with a Ca^{2+} chelator decreases the rate of milk secretion [16]. Lactating mammary glands also shut down the milk production process concurrent with the disruption of alveolar TJs after an injection of lipopolysaccharide [17, 18]. These findings suggest that the less permeable TJs are closely related to the milk production status of alveolar MECs.

A TJ is an intercellular junction localized at the apical-most region of the epithelial cells to regulate the flow of ions, water, and other small molecules through the paracellular pathway [19, 20]. The structure of TJs in the cell membrane consists of a continuous network of intramembranous strands that can be observed by freeze-fracture electron microscopy. TJ strands also restrict the lateral diffusion of membrane lipids and proteins between the apical and basolateral membranes to maintain cellular polarity [21-23]. The TJ strands are composed of one or more types of claudins (Cldns) [24]. Cldns are transmembrane proteins and their composition determines the network pattern of the TJ strands and the paracellular permeability of the TJs [25]. Furthermore, TJ strands form pores to allow ions and water to pass through, depending on the composition of the Cldn subtypes. Therefore, a

compositional change of Cldns reflects functional and structural changes of the TJs. In mammary glands, changes in TJ strand networks are observed around the time of parturition, during lactation, and after weaning concurrent with changes in TJ permeability [12]. TJs in alveolar MECs during lactation mainly consist of Cldn3, whereas TJs before parturition and after weaning contain Cldn4 in addition to Cldn3 [26]. Lactating MECs also show a restricted localization of Cldn3 at the apical-most regions, while Cldn3 in non-lactating MECs is located in both the lateral membrane and the apical-most regions [18, 26]. Thus, the concentration of Cldn3 at the apical-most regions and the downregulation of Cldn4 occur in alveolar MECs to form lactation-specific TJs.

Alveolar MECs initiate the production of milk components in response to glucocorticoids and Prl after parturition [1, 2]. Alveolar MECs also form less permeable TJs to prevent leakage of milk components and fluids across the alveolar lumen and the mammary interstitium during lactation [9-11]. Nguyen et al. suggested that glucocorticoids and Prl are necessary for TJ closure after parturition [27]. The glucocorticoid/GR complex functionally interacts with Stat5 to mutually regulate transcription [5]. These previous reports suggest that Prl/STAT5 and glucocorticoid/GR pathways are involved with the induction of milk production and the formation of less permeable TJs in alveolar MECs. However, it remains unclear whether the activation of Prl/STAT5 and glucocorticoid/GR pathways induces less

permeable TJs by the downregulation of Cldn4 and the concentration of Cldn3 at TJs in alveolar MECs in association with lactogenesis in MECs. In this study, we investigated the influence of Prl and glucocorticoids on the expression patterns of Cldn3 and Cldn4 with respect to TJ permeability and the milk production capacity in MECs.

2. Materials and methods

2.1. Animals

Virgin and pregnant female ICR mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) and were maintained under conventional conditions at 22–25°C. The virgin mice were used for *in vitro* experiments to isolate MECs, and the pregnant mice were used for *in vivo* experiments. After parturition, each lactating mouse was kept with more than 10 suckling pups. For preparation of the involuting mammary glands, we performed forced weaning by separation of the pups on day 10 of lactation. Subsets of mice were decapitated at the following time points: on day 17 of gestation (*pregnancy*), immediately after parturition (*parturition*), on day 10 of lactation (*lactation*), or on day 1 of forced weaning (*involution*). The mammary glands were then quickly dissected from decapitated mice. A Prl secretion inhibitor, bromocriptine (20 mg /kg), was administered intraperitoneally to the mice on day 10 of lactation. The mammary glands were dissected from decapitated mice 6, 12, or 24 h

after the administration of bromocriptine.

The dissected mammary glands were immersed in 4% formaldehyde in phosphate-buffered saline (PBS) for the preparation of paraffin sections, in an RNAlater solution for quantitative reverse-transcription PCR (qRT-PCR), or in a lysis buffer for western blotting. All experimental procedures in this study were approved by the Animal Resource Committee of Hokkaido University and were conducted in accordance with Hokkaido University guidelines for the care and use of laboratory animals.

2.2. Reagents

Prl from sheep pituitary (catalogue # L6520), Dex (cat. # D1756), and bromocriptine (cat. # B2134) were purchased from Sigma-Aldrich (St. Louis, MO). AG490 (JAK inhibitor; cat. # T9969) and RU486 (GR antagonist; cat. # M3321) were purchased from LKT Laboratories, Inc. (St. Paul, MN). The following antibodies served as primary antibodies in our immunoassays: rabbit polyclonal antibodies against Cldn3 (Life Technologies, Gaithersburg, MD, cat. # 34-1700), Cldn4 (Life Technologies; cat. # 36-4800), GR (Sigma-Aldrich; cat. # SAB4501309), STAT5 (Cell Signalling Technology, Danvers, MA; cat. # 9363), pSTAT5 (on Tyr694; Cell Signalling Technology, cat. # 4322), pSTAT5a (Abcam, Cambridge, UK; cat. # ab30648), α_{S1} -casein (Santa Cruz Biotechnology, Santa Cruz, CA; cat. # sc-98699); mouse

monoclonal antibodies against pan-keratin (Sigma-Aldrich; cat. # C2562) and occludin (OcIn; Life Technologies; cat. # 33-1500), and a goat polyclonal antibody against β -casein (Santa Cruz Biotechnology; cat. # sc-17969). The secondary antibodies (an Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody, Alexa Fluor 546-conjugated goat anti-mouse IgG antibody, and Alexa Fluor 546-conjugated donkey anti-goat IgG antibody) were purchased from Life Technologies for immunofluorescence staining. The secondary horseradish peroxidase (HRP)-conjugated anti-mouse, anti-rabbit, and anti-goat IgG antibodies for western blotting were purchased from Sigma-Aldrich.

2.3. Cell culture

MECs were isolated from the fourth mammary gland of virgin ICR mice. The minced mammary glands were incubated with the RPMI-1640 medium (GIBCO-BRL, Grand Island, NY) containing type III collagenase at 2 mg/mL (Worthington Biochemical Corporation, Lakewood, NJ) for 2 h at 37°C with gentle shaking, followed by treatment with 0.25% trypsin in RPMI-1640 for 5 min at room temperature. The fragments of the mammary epithelium were dissociated by gentle pipetting with a Pasteur pipette. After centrifugation, the pellet was resuspended in foetal bovine serum (FBS; GIBCO) and then centrifuged and resuspended two more times at 100 rpm for 5 min to separate the epithelial fragments. The

epithelial fragments were cultured in RPMI-1640 supplemented with 10% FBS, 10 µg/mL insulin (Sigma-Aldrich; cat. # I6634), 10 ng/mL epidermal growth factor (EGF; cat. # 354001, BD Biosciences, Bedford, MA), 100 U/mL penicillin (GIBCO), and 100 µg/mL streptomycin (GIBCO). After the MECs reached confluence, they were cultured for five additional days in RPMI-1640 supplemented with 1% FBS, 10 µg/mL insulin, 10 ng/mL EGF, 0.5 U/mL Prl, and 1 µM Dex. At the beginning of this period, 10 mM stock solutions of RU486 and AG490 that were dissolved in DMSO were added to the medium as inhibitors of glucocorticoids and JAK2, respectively. MECs were also cultured on a poly-L-lysine-coated cover glass for immunofluorescence staining.

2.4. Immunofluorescence staining

The formaldehyde-fixed mammary glands were embedded in paraffin and cut into 5-µm-thick slices. The paraffin sections were deparaffinized and hydrated in 10 mM Tris-HCl (pH 10.5) with 0.5 mM EGTA in distilled water for 20 min before antigen retrieval by microwave treatment. MECs cultured on poly-L-lysine-coated cover glass were fixed with methanol for 10 min at -20°C and then with 1% formaldehyde in PBS for 10 min at 4°C. The deparaffinized slices and fixed MECs were incubated with PBS containing 5% bovine serum albumin (BSA; Sigma-Aldrich) to block nonspecific interactions and were then incubated

with the primary antibody diluted in the blocking solution overnight at 4°C. After the slices and MECs were washed with PBS, they were exposed to an appropriate secondary antibody for 1 h at room temperature in the blocking solution. Control samples were processed in the same manner, except that the primary antibody was absent. Images of the stained slices and MECs were acquired using a confocal laser-scanning microscope (TCS SP5; Leica, Mannheim, Germany).

2.5. Western blotting analysis

Samples of the mammary glands and MECs were analysed using electrophoresis in a 7.2% or 12.5% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked for 1 h with PBS containing 4% nonfat dried milk and 0.05% Tween-20 and were then incubated overnight at 4°C with primary antibodies diluted in PBS containing 2.5% BSA. In the case of β -casein analysis, we used PBS containing 2% BSA as the blocking solution. Subsequently, the membranes were washed in PBS containing 0.05% Tween 20 and incubated for 45 min at room temperature with appropriate secondary HRP-conjugated antibodies diluted in PBS containing 3% nonfat dried milk and 0.05% Tween-20. The immunoreactive bands were detected using Luminate Forte Western HRP Substrate (Millipore, Billerica, MA). The

images of the protein bands were obtained with a ChemiDoc EQ densitometer and processed with Quantity One software (Bio-Rad).

2.6. qRT-PCR analysis

Total RNA from the mammary glands was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA). Reverse transcription was performed using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). The qRT-PCR was conducted on a Light Cycler 480 (Roche Applied Science, Indianapolis, IN) with Thunderbird SYBR qPCR Mix (Toyobo). We used the following cycling conditions: 95°C for 1 min followed by 40 cycles at 95°C for 15 s and 58°C for 1 min. The primers were as follows: *β-casein* (*Csn2*)-specific primers, 5'-CTTCAGAAGGTGAATCTCATGGG-3' (forward) and 5'-CAGATTAGCAAGACTGGCAAGG-3' (reverse); *κ-casein* (*Csn3*)-specific primers, 5'-TCGACCCCATTACTCCCATTGTGT-3' (forward) and 5'-TGTAAGGTAAGGGAAGACGAGAAAGAT-3' (reverse); *Cldn3*-specific primers, 5'-AGCCAGTCTCCAAAGCCACA-3' (forward) and 5'-CTGGGAATCAACTGCCCTTC-3' (reverse); *Cldn4*-specific primers, 5'-TGGCGTCTATGGGACTACAGG-3' (forward) and 5'-GATGTTGCTGCCGATGAAGG-3' (reverse); and *Gapdh*-specific primers, 5'-GAGCGAGACCCCACTAACATC-3' (forward) and 5'-GCGGAGATGATGACCCTTTT-3' (reverse).

(reverse). *Gapdh* served as an internal control.

2.7. Measurement of TJ permeability

To measure transepithelial resistance (TER), MECs were cultured on a cell culture insert in a 24-well plate (0.4- μ m pore size; BD Biosciences). The electrodes of a Millicel-ERS system (Millipore) were placed in the upper and lower chambers, and TER was measured. The TJ permeability of MECs was also evaluated by measuring the flux of fluorescein. The upper chamber of the insert was filled with a medium containing 0.1 mg/mL fluorescein sodium salt (molecular weight 376; Sigma-Aldrich), and the lower chamber was filled with just the medium. Medium from the lower chamber was collected 30 min after the addition of tracers, and the paracellular flux was measured using a fluorimeter (excitation 492 nm, emission 520 nm).

2.8. Statistical analysis

The data were expressed as the mean (standard error of the mean [SEM]). Significance values were calculated by Bonferroni corrected two-tailed Student's *t*-test following one-way analysis of variance (ANOVA). Differences were considered significant at *p* values <0.05 and <0.005, indicated by asterisks or lowercase letters. All experiments were performed a

minimum of four times using different samples.

3. Results

3.1. Temporal changes in Cldn3 and Cldn4 along with activation of the Prl/STAT5 and glucocorticoid/GR pathways

The expression patterns of Cldn3 and Cldn4 change during pregnancy, lactation and involution in alveolar MECs in mammary glands [26]. We investigated whether the changes in Cldn3 and Cldn4 expression occur in conjunction with activation/inactivation of the Prl/STAT5 and glucocorticoid/GR pathways. During the pregnancy and involution periods, Cldn3 was localized in the basolateral membranes and at the apical-most regions, as determined by the localization of Ocln, which is a representative TJ component (Fig. 1A) [28]. During lactation, alveolar MECs showed localization of Cldn3 at the apical-most regions, along with Ocln, and was not localized in the basolateral membrane. Cldn4 was almost undetectable in alveolar MECs during lactation, although alveolar MECs clearly expressed Cldn4 during the pregnancy and involution periods at the apical-most regions and in the lateral membrane, in line with our previous report [26]. In addition, the mRNA expression of *Cldn4* was significantly lower during lactation, while *Cldn3* did not show significant expressional changes throughout pregnancy, lactation and involution (Fig. 1B).

Activation of the glucocorticoid/GR and Prl/STAT5 pathways was confirmed by the localization of GR and pSTAT5a in the nuclei [29]. Most of the alveolar MECs were positive for GR and pSTAT5a in their nuclei during lactation (Fig. 1A, C). In late pregnancy, GR and pSTAT5a immunostaining signals were weak, and the percentage of GR- and pSTAT5a-positive alveolar MECs was 25% and 39%, respectively. Alveolar MECs showed little or no GR or pSTAT5a immunostaining in their nuclei on day 1 of involution. Western blotting analysis also showed upregulation of pSTAT5 and GR during lactation (Fig. 2D). In addition, we determined the expression of β -casein (*Csn2*), which is the major milk protein induced after lactogenesis [30]. The expression of β -casein gradually increased during the pregnancy, parturition and lactation periods (Fig. 1E). However, mammary glands had minimal β -casein expression after weaning. These results suggest that the expression patterns of *Cldn3* and *Cldn4* in MECs are associated with milk production and the activation/inactivation of the glucocorticoid/GR and Prl/STAT5 pathways.

3.2. Prl and Dex modulate the expression of Cldn3 and Cldn4 along with lactogenesis

To investigate whether activation of the Prl/STAT5 and glucocorticoid/GR pathways is involved in the expression patterns of *Cldn3* and *Cldn4*, MECs were cultured in the presence of dexamethasone (Dex), which is a glucocorticoid analogue, and Prl. After 5 days

of treatment, MECs showed a cobblestone-like appearance regardless of the presence of Prl or Dex, although the outlines of MECs treated with Prl were more rounded than those treated with Dex (Fig. 2A). In the absence of Prl and Dex, the nuclei of the MECs were pSTAT5a- and GR-negative (Fig. 2A). Prl and Dex induced the nuclear localization of pSTAT5a and GR, respectively. Simultaneous treatment with Prl and Dex induced nuclear localization of both pSTAT5a and GR, and the percentage of GR- and pSTAT5a-positive MECs was 87% and 98%, respectively (Fig. 2B). Western blotting analysis also showed upregulation of pSTAT5 and GR with Prl and Dex treatments, respectively (Fig. 2C).

The milk production capacity of MECs was evaluated based on the expression and secretion of β -casein, which is one of the major milk-specific proteins. Immunofluorescence staining showed that β -casein-positive cells were observed in the presence of Prl and were increased by co-treatment with Prl and Dex (Fig. 3A). MECs treated with Dex in the absence of Prl did not show intracellular localization of β -casein, which was similar to what was observed in the control cells. Intracellular β -casein was detected in MECs treated with Prl by western blotting, although secreted β -casein was at an almost undetectable level (Fig. 3B). Simultaneous treatment with Prl and Dex drastically increased both intracellular and secreted β -casein (Fig. 3C). Induction of β -casein expression by Prl treatment was confirmed by qRT-PCR. Cotreatment with Prl and Dex upregulated the expression of

β -casein in MECs by approximately 10-fold compared with that in MECs treated with only Prl (Fig. 3D). These results suggest that treatment with Prl and Dex induced substantial milk production in MECs.

Treatment with Prl and Dex influenced the expression patterns of Cldn3 and Cldn4. In the absence of Prl or Dex, Cldn3 was localized along the apical-most regions, as represented by the localization of OcIn, with some interruptions and was only occasionally present in the basolateral membrane (Fig. 4A). Dex treatment in the absence of Prl induced continuous localization of Cldn3, along with OcIn, along the apical-most regions and in the basolateral membrane of MECs. In contrast, Prl treatment caused disruption of the network-like localization pattern of Cldn3 and OcIn. This disruption did not occur in the presence of Dex even when Prl was present in the MEC culture medium. In the presence of Dex and Prl, Cldn3 was concentrated along the apical-most regions together with OcIn. In the absence of Prl and Dex, Cldn4 was localized in the basolateral membrane and only occasionally at the apical-most regions, together with OcIn. The localization of Cldn4 in the basolateral membrane was also clearly observed in MECs treated with Dex without Prl. In the presence of Prl, Cldn4-positive regions drastically decreased in MECs. After cotreatment with Prl and Dex, Cldn4 was almost undetectable in the basolateral membrane and was only slightly localized at the apical-most regions with OcIn, similar to the pattern seen in lactating

alveolar MECs *in vivo* (Fig. 1).

Western blotting analysis showed decreases in *Cldn3* and *Cldn4* in MECs after Prl treatment (Fig. 4B). In contrast, Dex increased *Cldn3* and *Cldn4* in a time dependent manner (Fig. 4C). Cotreatment with Prl and Dex decreased *Cldn4* by almost half, while *Cldn3* did not show significant decreases over 5 days of treatment (Fig. 4D). Treatment of Prl in the presence of 100 nM Dex resulted in a greater decrease in *Cldn4* than in *Cldn3* in a dose-dependent manner (Fig. 4E). A high concentration of Prl (2.5 U /mL) along with Dex upregulated *Cldn3* but downregulated *Cldn4*. AG490, which blocks the Prl/STAT5 pathway by inhibiting JAK2, caused a significant upregulation of *Cldn4* in the presence of Prl and Dex (Fig. 4F). However, RU486, which blocks the binding of glucocorticoids to the GR, significantly downregulated *Cldn3* to a greater extent than it did *Cldn4*. Simultaneous application of AG490 and RU486 significantly upregulated *Cldn4*, while *Cldn3* expression was downregulated. The results of qRT-PCR also showed a Prl-induced downregulation and a Dex-induced upregulation of *Cldn3* and *Cldn4* in MECs (Fig. 4G). Cotreatment with Prl and Dex decreased the expression of *Cldn4* but not *Cldn3*.

3.3. Dex-induced formation of less permeable TJs regardless of the presence of Prl

The permeability of TJs was evaluated by measuring TER and fluorescein flux through the

paracellular pathway in MEC sheets. TER is a nearly instantaneous electrical assessment of ionic permeation through paracellular pathway, and measurement of fluorescein flux allows quantification of solute flux over longer time periods [31-33]. Dex increased the electric resistance of the MEC sheet and decreased the flow of fluorescein through the paracellular pathway in the absence of Prl (Fig. 5A, B). In contrast, Prl treatment decreased the electric resistance and increased the flow of fluorescein in comparison to treatment with the vehicle or Dex. Cotreatment with Dex and Prl induced an increase in TER and a decrease in fluorescein flux similar to treatment with Dex alone.

3.4. An inhibitor of Prl secretion changed the expression patterns of Cldn3 and Cldn4, with inactivation of the Prl/STAT5 and glucocorticoid/GR pathways

To assess the influence of Prl on alveolar MECs *in vivo*, we administered bromocriptine, which inhibits Prl secretion and reduces the level of Prl in the blood, to the lactating mice [34]. Bromocriptine weakened the positive reaction to pSTAT5a in the nuclei of alveolar MECs 12 h after administration (Fig. 6A). A decrease in pSTAT5 was also detected by western blotting analysis, suggesting that inactivation of the Prl/STAT5 pathway occurred with bromocriptine treatment (Fig. 6B). pSTAT5a-positive cells partially recovered 24 h after administration. In addition, the immunostaining intensity and western blotting bands of GR

were weak 12 and 24 h after the administration of bromocriptine. In alveolar MECs, intracellular α_{s1} -casein was almost undetectable 12 and 24 h after the administration of bromocriptine, although α_{s1} -casein accumulation in the alveolar lumen was observed. The expression of β -casein and κ -casein mRNA also significantly decreased after the administration of bromocriptine (Fig. 6C)

The administration of bromocriptine influenced the expression patterns of Cldn3 and Cldn4. Alveolar MECs showed localization of Cldn3 in the lateral membranes and at the apical-most regions, as indicated by localization of Ocln, 24 h after bromocriptine administration (Fig. 7A). In the vehicle control and in normal lactating mammary glands, Cldn3 was localized only at the apical junctions (Fig. 1). Cldn4 was also observed in the basolateral membranes in alveolar MECs 24 h after the administration of bromocriptine, whereas vehicle control samples showed only minimal localization of Cldn4 (Fig. 7B). The gradual increases in Cldn3 and Cldn4 after administration of bromocriptine were confirmed by western blotting analysis (Fig. 7C). Densitometry analysis showed a 2-fold and 18-fold upregulation of Cldn3 and Cldn4, respectively (Fig. 7D). The results of qRT-PCR also showed a significant increase in *Cldn4* in mammary glands 24 h after the administration of bromocriptine (Fig. 7E).

4. Discussion

Alveolar MECs in lactating mammary glands synthesize and secrete milk components to supply milk to suckling infants. Lactating MECs also have less permeable TJs to prevent the leakage of milk components [9-11]. Less permeable TJs are formed after parturition in association with the downregulation of Cldn4 and an increase in the concentration of Cldn3 at the apical-most regions in MECs [35]. In the present study, we focused on Prl and glucocorticoids as key regulators that induce less permeable TJs because the Prl/STAT5 and glucocorticoid/GR pathways are involved in the induction of lactogenesis in mammary glands around the time of parturition [36]. Our results showed distinct and synergetic roles of the Prl/STAT5 and glucocorticoid/GR pathways in lactation-specific TJ formation and lactogenesis in MECs.

Prl disrupted the linear localization of Cldn3 and Cldn4 along the apical-most regions by downregulating their expression in MECs. The Prl-induced TJs in MECs were significantly looser than those in untreated MECs. Inhibition of the Prl/STAT5 pathway by AG490 induced a drastic upregulation of Cldn4 in MECs treated with Prl and Dex *in vitro*. In addition, inhibition of Prl secretion by bromocriptine upregulated Cldn3 and Cldn4 and was associated with a reduction in milk secretion from alveolar MECs *in vivo*. The adverse effects of Prl on the TJs in MECs occurred simultaneously with the activation of the

Prl/STAT5 pathway. The milk production status of alveolar MECs is maintained via the Prl/STAT5 pathway, and inactivation of the pathway shuts down the milk production [17, 37, 38]. In this study, intracellular β -casein was detected in Prl-treated MECs along with the activation of STAT5. Thus, the Prl/STAT5 pathway induces milk production while TJs become looser simultaneously with (or because of) downregulation of Cldn3 and Cldn4.

In contrast to Prl, Dex, a glucocorticoid analogue, induced the upregulation of Cldn3 and Cldn4 concurrent with the formation of less permeable TJs. Inhibition of the glucocorticoid/GR pathway by RU486 caused the downregulation of Cldn3 and Cldn4. Glucocorticoids are well known regulators of TJs in several epithelial tissues, including the intestines [39], trachea [40], amnion [35], and skin [41]. Glucocorticoids also stimulate the formation of less permeable TJs through a downregulation of RhoA in the mammary epithelium [42-45]. In this study, Dex concentrated the localization of Cldn3 and Cldn4 at the apical-most regions (TJ regions) *in vivo* and *in vitro*. However, Dex treatment induced minimal expression or secretion of caseins in MECs in the absence of Prl, even though the glucocorticoid/GR pathway was activated. These results show that the glucocorticoid/GR pathway does not induce milk secretion and that TJs are made less permeable through the upregulation of Cldn3 and Cldn4.

Individual treatment with Prl and Dex induced milk production and the formation of less

permeable TJs in MECs, respectively. However, the milk production capacity induced by Prl treatment was relatively weak in the absence of Dex. In addition, TJs induced by Dex treatment in the absence of Prl consisted of both Cldn3 and Cldn4, while the TJs in normal lactating mammary glands contained almost no Cldn4. However, simultaneous treatment with Prl and Dex induced both a high level of milk production and less permeable TJs without Cldn4 in MECs. Stat5 and the GR form a molecular complex, which participates in the induction of transcription of the β -casein gene [7, 46-48]. The synergistic effect of Prl and glucocorticoids has also been reported for whey acidic protein in alveolar MECs [49]. According to the present study, pSTAT5a and GR are both localized in the nuclei of MECs after Prl and Dex treatment. These findings suggest that activation of the glucocorticoid/GR pathway acts as a transcriptional coactivator of Stat5 and contributes to the enhancement of milk production along with the downregulation of Cldn4 for the formation of lactation-period specific TJs.

In normal lactating mammary glands, alveolar TJs are composed of Cldn3 and contain almost no Cldn4. However, Cldn4 is abundantly present in leaky TJs in the alveolar MECs before parturition and after weaning [9-11]. Inflammation of the mammary glands causes an upregulation of Cldn4 and a disruption of less permeable TJs in alveolar MECs during lactation in association with the inactivation of STAT5 [17, 18]. The composition of Cldns is a

key factor in determining the permeability of TJs [50]. Therefore, we assumed that the presence of Cldn4 in the alveolar TJs would increase their permeability. However, TJs containing both Cldn3 and Cldn4, induced by Dex treatment, were as less permeable as TJs containing only Cldn3, induced by the simultaneous treatment of Prl and Dex. The permeability of alveolar TJs may be regulated by the completeness of TJ sealing between MECs because MECs without Dex treatment show discontinuous TJ networks along the apical-most regions together with leaky TJs. Furthermore, the interactions of Cldn3 and Cldn4 with other Cldn subtypes may regulate the permeability of TJs. Lactating mammary glands express Cldn1, 2, 3, 4, 5, 7, 8, 15, and 16 [51]. The composition of Cldns determines the paracellular permeability of TJs because the extracellular loops of Cldns from adjacent cells interact with each other to regulate paracellular transport between the luminal and basolateral spaces [25, 52]. For example, Cldn4 interacts with Cldn8 to form an anion-selective paracellular pathway in kidney collecting duct [53]. Cldn4 is observed in both less permeable and leaky TJs in different epithelial tissues, which exhibit distinct Cldn composition [54-59]. Cldns also form multiprotein complex with TJ-related proteins such as junctional adhesion molecule A (JAM-A) and zonula occludens protein-1 (ZO-1) to regulate the permeability of TJs [60-63]. In particular, mRNA of ZO-1, which down-regulates after weaning together with Cldn3, is essential for TJ assembly in MECs [64, 65]. Therefore, it is

possible that other Cldn subtypes and TJ-related proteins regulate the permeability of TJs in alveolar MECs.

In summary, our results show that Prl and Dex induce milk production and the formation of less permeable TJs among MECs, respectively, and that Prl and Dex synergistically enhance milk production in MECs simultaneously with the formation of lactation-period specific less permeable TJs. During lactation, the Prl/STAT5 and glucocorticoid/GR pathways are activated in alveolar MECs, suggesting that coactivation of the Prl/STAT5 and glucocorticoid/GR pathways successfully drives both active milk production and the formation of less permeable TJs (Fig. 8). However, multiple factors such as serotonin, insulin-like growth factor-I, and lipopolysaccharide have been reported to change the TJ permeability in MECs[18, 45, 66, 67]. The barrier function in alveolar MECs may be more intricately regulated during lactation.

Author contributions

K.K. conceived the study, participated in the research design and implementation of the study, analysed and interpreted the data, and drafted the manuscript. Y.T., K.M., C.K. and S.O. performed the experiments and analysed the data. H.K. interpreted the data and helped to draft the manuscript. All authors read and approved the final manuscript.

Disclosure statement

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Figure legends

Fig. 1

Changes in the expression of *Cldn3* and *Cldn4* along with activation of the *Prl/STAT5* and glucocorticoid/*GR* pathways in mouse mammary glands

(A) Mammary glands during late pregnancy, lactation, and involution periods were immunostained with anti-*Cldn3* (green), anti-*Cldn4* (green), anti-pSTAT5a (red), and anti-GR (green) antibodies. Nuclei stained with 4',6-diamidino-2-phenylindole (DAPI) are blue, and the red staining in the images related to *Cldn3* and *Cldn4* represents OcIn, which indicates areas with TJs in the apical-most regions of the lateral membranes in alveolar MECs. The scale bars represent 20 μ m. (B) The expression of *Cldn3* and *Cldn4*, determined by qRT-PCR. The data are presented as the mean (SEM); $n = 6$. Different lowercase letters show significant differences within each *Cldn*; $p < 0.05$. (C) The percentage of pSTAT5a- and GR-positive cells in the nuclei among all alveolar MECs. The data are presented as the mean (SEM); $n = 10$. Different lowercase letters show significant differences within pSTAT5a and GR, respectively; $p < 0.05$. Different lowercase letters show significant differences; $p < 0.05$. (D) The results of western blotting analysis of pSTAT5, STAT5, GR and β -actin. (E) The expression of β -casein, determined by qRT-PCR. The data are presented as the mean (SEM); $n = 6$. Different lowercase letters show significant differences; $p < 0.05$.

Fig. 2

Prl and Dex activate the Prl/STAT5 and glucocorticoid/GR pathways in MECs

MECs were cultured with or without Prl (0.5 U/mL) and Dex (1 μ M) for 5 days. (A) Phase-contrast microscopic images and immunostaining images of GR (green) with pan-keratin (red), and pSTAT5a (green). Nuclei stained with DAPI are blue. The scale bars represent 10 μ m. (B) The percentage of pSTAT5a- and GR-positive cells in the nuclei among all MECs. The data are presented as the mean (SEM); n = 4. (C) The results of western blotting analysis of pSTAT5, STAT5, GR and β -actin.

Fig. 3

Cotreatment with Prl and Dex enhances expression and secretion of β -casein in MECs

MECs were cultured with or without Prl (0.5 U/mL) and Dex (1 μ M) for 5 days. (A) Immunostaining images of β -casein (red) with nuclei staining with DAPI (blue). The scale bars represent 10 μ m. (B, C) The results of western blotting and densitometry analysis of intracellular and secreted β -casein with β -actin used as an internal control; n = 4. Different lowercase letters show significant differences among the treatments within intracellular and secreted β -casein, respectively; $p < 0.05$. (D) The expression of β -casein in MECs,

determined by qRT-PCR. The data are presented as the mean (SEM); $n = 4$. Different lowercase letters show significant differences among the treatments; $p < 0.05$.

Fig. 4

Prl and Dex influence the expression patterns of *Cldn3* and *Cldn4* in MECs

(A) Immunostaining images of *Cldn3* (green) and *Cldn4* (green) with *Ocln* (red) in MECs cultured with or without Prl (0.5 U/mL) and Dex (1 μ M) for 5 days. Nuclei stained with DAPI are blue. The scale bars represent 10 μ m. (B-F) The results of western blotting and densitometry analysis of *Cldn3* and *Cldn4* with β -actin used as an internal control. MECs were cultured for 5 days with or without Prl (0.5 U/mL or 0–2.5 U/mL) and Dex (1 μ M). MECs were pretreated with 10 μ M AG490 (JAK inhibitor) and 5 μ M RU486 (glucocorticoid receptor antagonist) 1 h before Prl (0.5 U/mL) and Dex (100 nM) treatment. The data are presented as the mean (SEM); $n = 5$; $*p < 0.05$ and $**p < 0.005$. (G) The expression of *Cldn3* and *Cldn4*, determined by qRT-PCR. The data are presented as the mean (SEM); $n = 6$; $*p < 0.05$ and $**p < 0.005$.

Fig. 5

Prl and Dex influence the permeability of TJs in MECs

We measured transepithelial resistance (TER) (A) and fluorescein flux (B) through MEC

sheets in the cell culture inserts in the presence or the absence of Prl (0.5 U/mL) and Dex (1 μ M) for 5 days. The data are presented as the mean (SEM); n = 6. Different lowercase letters show significant differences among the treatments for each time point; $p < 0.005$.

Fig. 6

The influence of bromocriptine on the Prl/STAT5 and glucocorticoid/ GR pathways and milk production in lactating mammary glands

Bromocriptine, a Prl secretion inhibitor, was administered to lactating mice 10 days after parturition. (A) The mammary glands were immunostained with anti-pSTAT5a, anti-GR, and anti- α_{S1} -casein antibodies. Nuclei stained with DAPI are blue. The scale bars represent 20 μ m. (B) Results of western blotting of pSTAT5, STAT5, GR, β -casein, and β -actin in mammary glands: vehicle administration and 6, 12, and 24 h after bromocriptine administration. (C) The expression of β -casein and κ -casein, determined by qRT-PCR. The data are presented as the mean (SEM); n = 4. Asterisks show significant differences within each casein compared with vehicle; $*p < 0.005$.

Fig. 7

The influence of bromocriptine on the expression patterns of Cldn3 and Cldn4 in lactating

mammary glands

(A, B) Lactating mammary glands 24 h after the administration of the vehicle or bromocriptine were immunostained with anti-Cldn3 (green; A), anti-Cldn4 (green; B), and anti-Ocln (red) antibodies. Nuclei stained with DAPI are blue. The scale bars represent 20 μ m. (C) The results of western blotting analysis of Cldn3, Cldn4, and β -actin in mammary glands: vehicle administration and 6, 12 and 24 h after bromocriptine administration. (D) The relative expression of Cldn3 and Cldn4 was measured by densitometry of the western blotting bands. The data are presented as the mean (SEM); n = 6. Asterisks show significant differences within each Cldn compared with vehicle; * p < 0.05 and ** p < 0.005. (E) The expression of *Cldn3* and *Cldn4*, determined by qRT-PCR. The data are presented as the mean (SEM); n = 4. Asterisks show significant differences within each *Cldn* compared with vehicle; * p < 0.05.

Fig. 8

The roles of Prl and Dex on inducing less permeable TJs and milk production in alveolar MECs

(A) In the absence of Prl and Dex, a glucocorticoid analogue, MECs are in a non-lactating state and have permeable TJs consisting of Cldn3 (red square) and Cldn4 (pink circle). (B)

In the presence of Dex, MECs are in a non-lactating state and have less permeable TJs consisting of large amounts of Cldn3 and Cldn4. (C) In the presence of Prl, MECs are in a low-lactating state and have permeable TJs consisting of small amounts of Cldn3 and Cldn4. (D) In the presence of Prl and Dex, MECs are in an high-lactating state and have less permeable TJs consisting of Cldn3. The arrows indicate the fluid flow through paracellular pathway regulated by TJs.

Figure 1

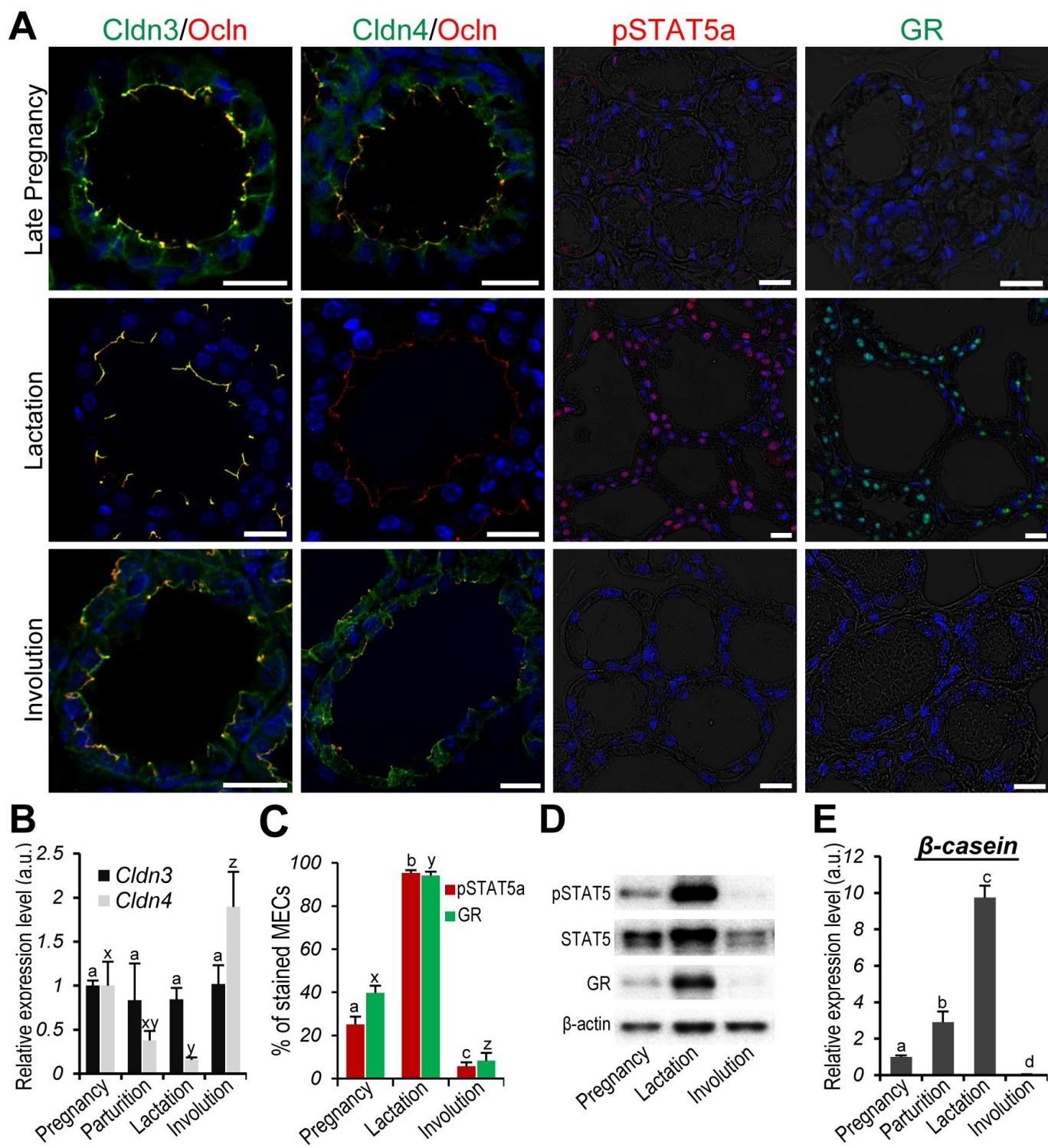


Figure 2

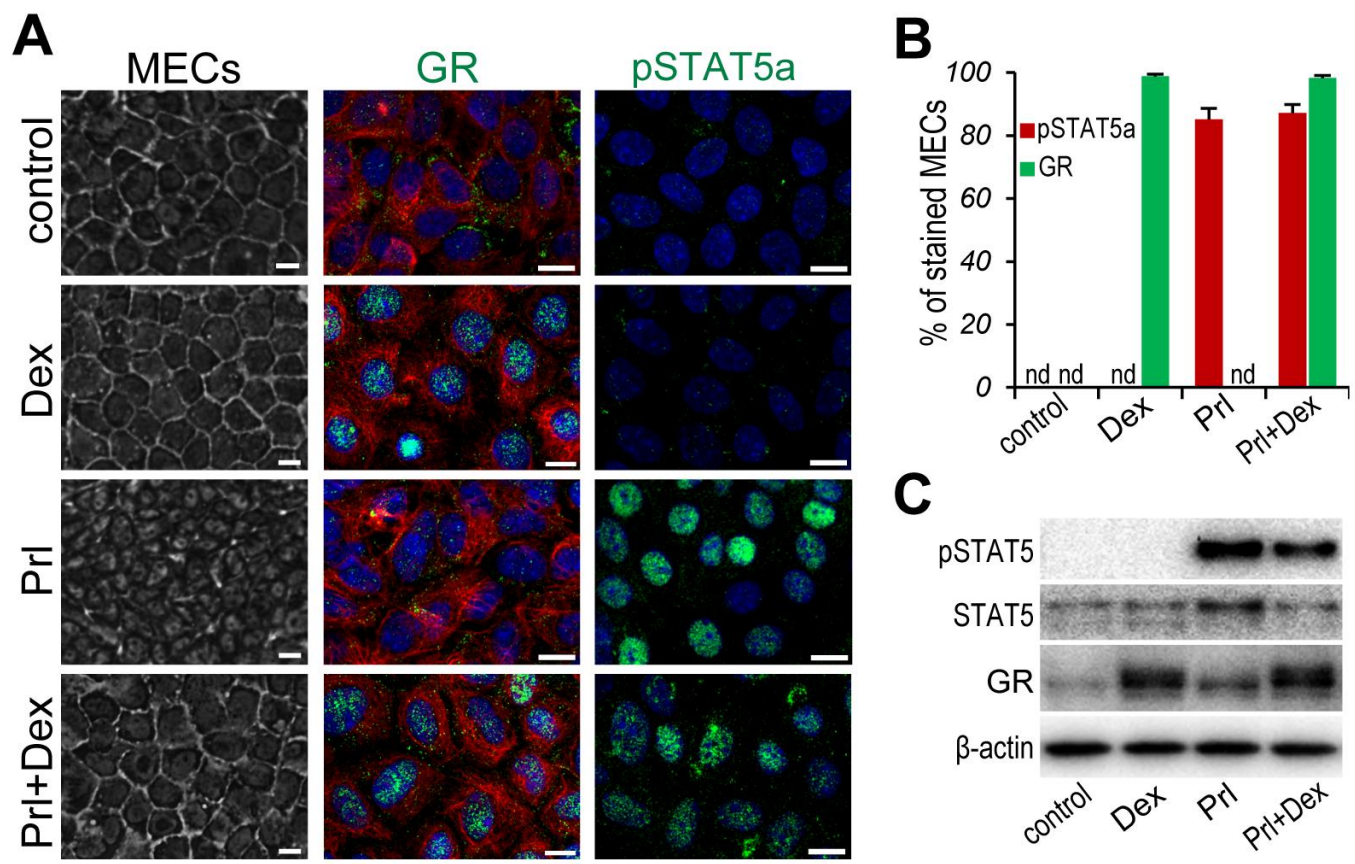


Figure 3

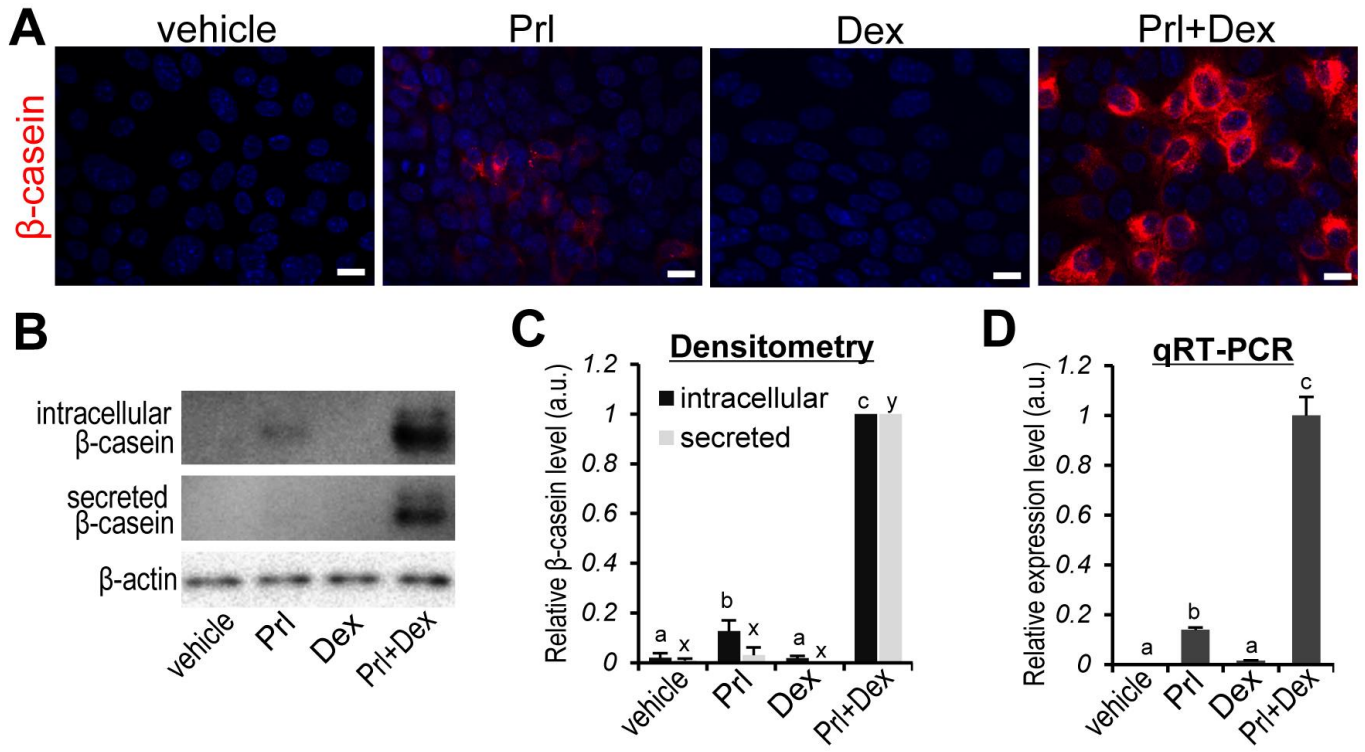


Figure 4

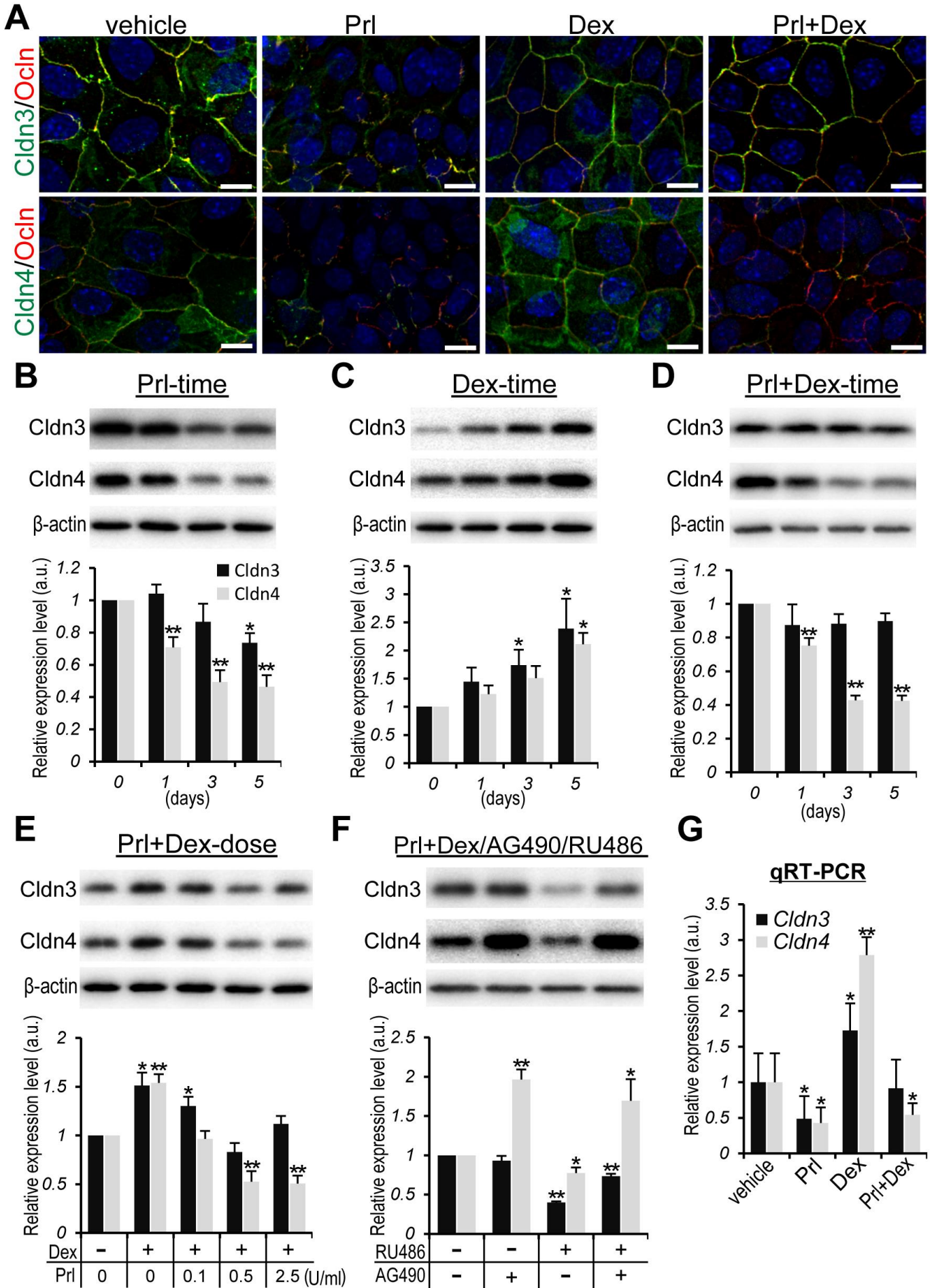


Figure 5

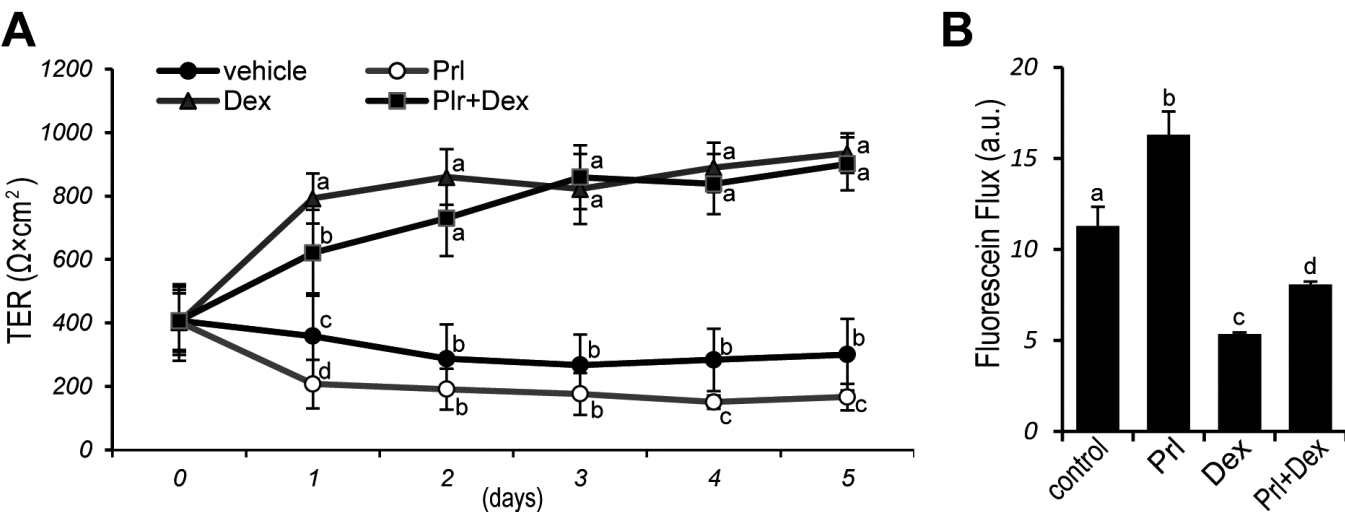


Figure 6

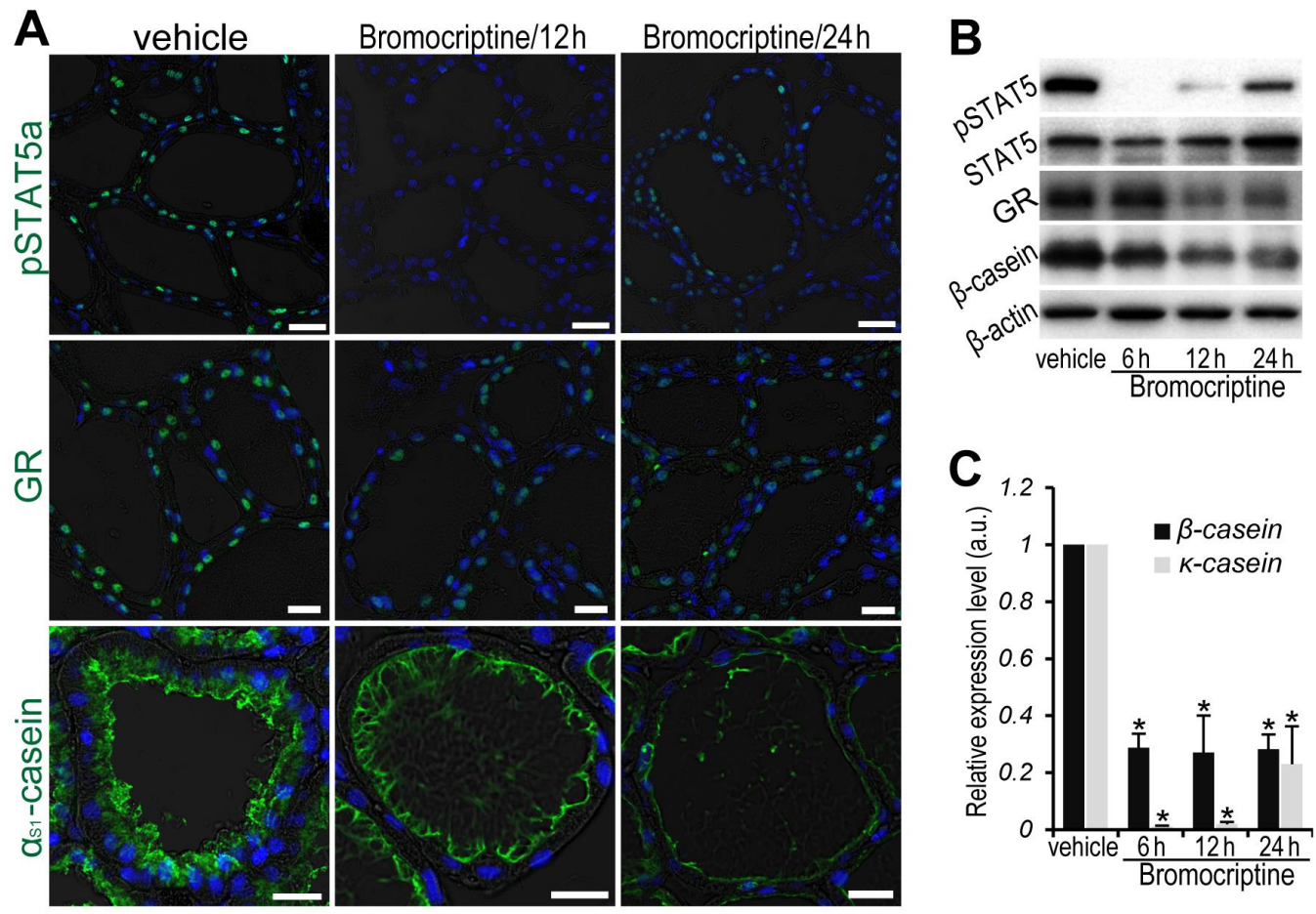


Figure 7

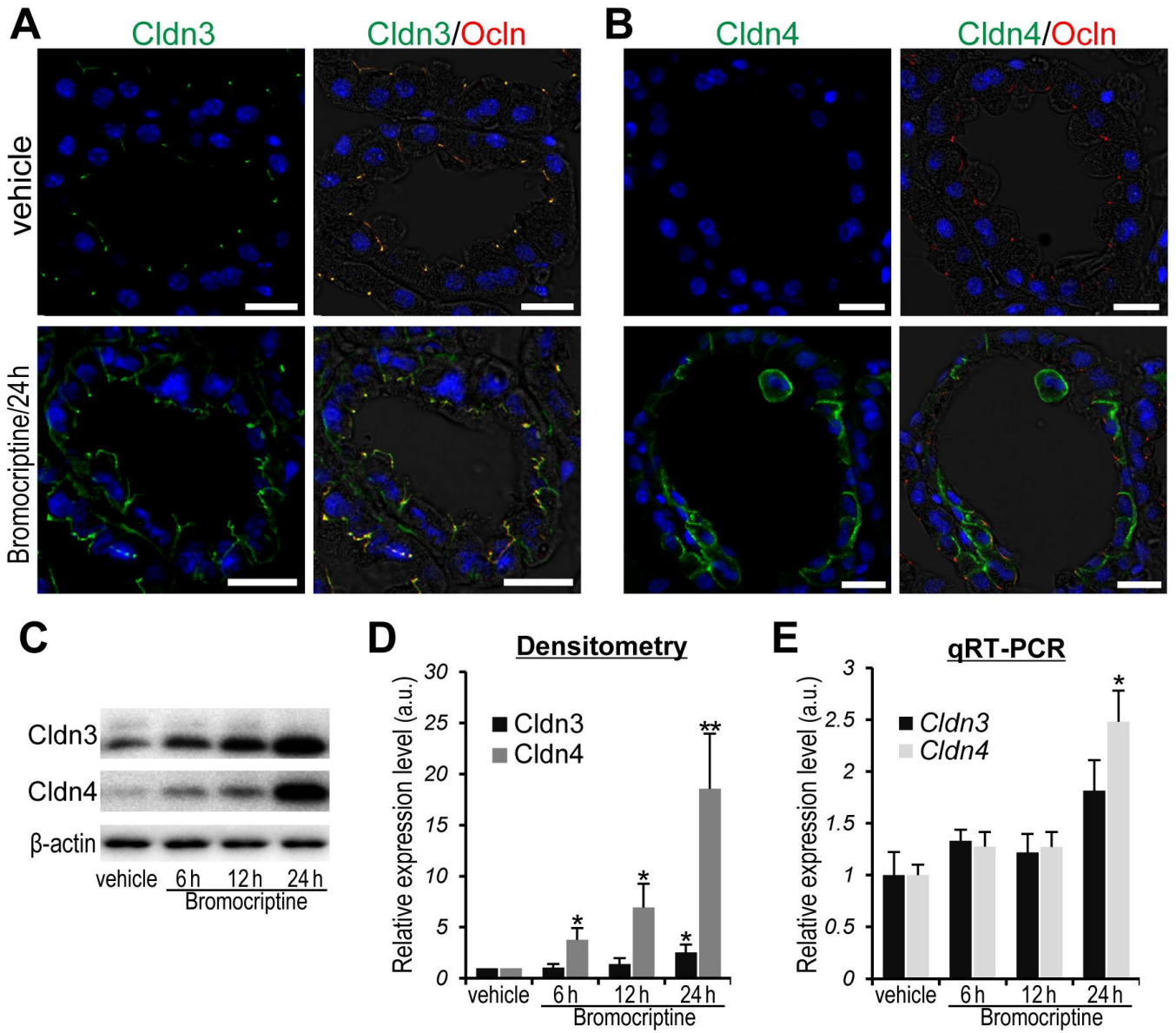


Figure 8

