Expression of tight junction molecule “claudins” in the lower oviductal segments and their changes with egg-laying phase and gonadal steroid stimulation in hens

Bambang Ariyadi, Naoki Isobe, Yukinori Yoshimura*
Graduate School of Biosphere Science, Hiroshima University, Higashi-Hiroshima, Japan

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Abstract
Tight junctions in the mucosal epithelium have essential roles as a mucosal barrier to prevent invasion of microbes into the hen oviduct tissue. The aim of this study was to determine the effects of the egg-laying phase and estradiol on the expression of tight junction molecule “claudins” in the lower oviductal segments in hens. White Leghorn laying and molting hens were used. Molting hens were given either sesame oil (vehicle) or estradiol benzoate (N = 5 per group) via injection. The lower segments of oviduct (isthmus, uterus, and vagina) of these birds were collected. Gene expression of claudin-1, -3, -5, lipopolysaccharide-induced TNFα factor (LITAF), and IFNα was analyzed by quantitative reverse transcription polymerase chain reaction, and localization of claudin-1 was examined by immunohistochemistry. Permeability in the mucosal epithelium was assessed by intrauterine injection of fluorescein isothiocyanate-dextran. Expression of claudin-1, -3, and -5 genes and density of claudin-1 protein in the lower oviductal segments were higher in laying hens than in molting hens (P < 0.01); their expression was upregulated by estradiol (P < 0.01). Expression of LITAF and IFNα genes was higher in molting hens than in laying hens. More fluorescein isothiocyanate-dextran infiltrated into the intercellular space of the uterus mucosal epithelium in molting hens than in laying hens and estradiol-treated molting hens. In conclusion, we inferred that barrier functions of the mucosal epithelium in the lower oviductal segments might be disrupted because of reduced claudin expression in molting hens, which might increase the susceptibility of mucosal tissue during the molting phase.

1. Introduction

In hens, microorganisms colonizing the cloaca might ascend the oviduct through the vagina and uterus. Mucosal barrier systems formed by epithelial cell junctions, mucus layer, and leukocyte activity, all have primary roles to prevent infection. Tight junctions (TJ) of mucosal epithelium are responsible for a paracellular barrier that protects underlying tissue against most toxic materials or pathogens [1–3]. Members of the claudin protein family form the main components of TJ; each family member laterally interacts with another claudin protein located in the adjacent cell in a homotypic or heterotypic manner [4,5]. They assemble in the plasma membrane, together with other proteins of the TJ complex, e.g., occludin and tricellulin [2,6]. Currently, nucleotide sequences of 3 claudins (claudin-1, -3, and -5) have been reported and 15 claudins (claudin-2, -4, -8, -10 to -12, -14 to -20, and -22 to -23) are predicted in the database of the National Center of Biotechnology Information [7]. Claudin-1 was present in high-resistance epithelia and crucial for the epidermal barrier. Claudin-3 was present in the tighter segment of the nephrone, whereas claudin-5 constituted TJ strands in endothelial cells [8,9]. Thus, claudin-1, -3, and -5 are likely to be expressed to form TJs in
epithelial tissues. In birds, the level of claudin-1 mRNA in immature testes was higher than that of adult testes of pheasant (*Phasianus colchicus*) [10]. Furthermore, expression of claudin-3, -5, and -16 in the intestinal epithelium was higher in 2-day-old chicks than in 20-day-old embryos [11]. Thus, claudin synthesis might be affected by physiologic and environmental factors.

Previous reports suggested that the oviduct was more susceptible in molting hens than laying hens because contamination of eggs by *Salmonella* was more frequent after resumption of laying in postmolting hens [12]. If expression of claudins in the oviducal epithelium declines, mucosal barrier functions might be weakened, leading to increased susceptibility to pathogenic agents. Proinflammatory cytokines such as TNFα and IFNγ downregulated expression of claudins and increased paracellular permeability in the epithelium [13–15]. We reported a reduction of mucin expression in the oviduct during the molting phase, which might reduce the mucin barrier on the tissue surface [16]. However, it remains unknown whether expression of claudin in the oviduct changes during the molting phase, compared with that in the laying phase. It is also unknown whether expression of TJ-related molecules is regulated by estrogen, although the growth of the oviduct is upregulated by this steroid [17].

The goals of this study were to determine differences in expression of claudins in the lower oviducal segments (vagina, uterus, and isthmus) during various egg-laying phases, namely, laying and molting, and the role of estradiol in their expression in hens. Specific objectives were to determine whether: (1) expression of claudin-1, -3, and -5, (2) expression of lipopolysaccharide-induced TNFα factor (LITAF) and IFNγ, and (3) permeability of fluorescein isothiocyanate (FITC)-dextran within mucosal epithelium in the lower oviducal segments, differed between laying and molting phases and if they were affected by estrogen stimulation.

# 2. Materials and methods

## 2.1. Experimental birds

Healthy White Leghorn laying and molting hens of approximately 500 days old were kept in individual cages under a 12-h light regimen of 14 hours light: 10 hours dark. Hens regularly laying four or more eggs in a clutch were provided with feed and water *ad libitum*. Molting hens were given restricted feed (25 g/d) and free access to water, which induced cessation of egg-laying after 5 to 7 days. They were used after 20 days of cessation of egg-laying as the molting hen group. A proportion of the molting hens were given 1 mg 17β-estradiol benzoate (Sigma-Aldrich Co., St. Louis, MO, USA; EB group) or 100 µL of sesame oil (control group) im once daily for 7 days. The birds were handled in accordance with regulations of Hiroshima University for animal experiments.

## 2.2. Analysis of claudin and cytokine expression

Anesthesia was induced with Somnopentyl (Kyoritsu Seiyaku Inc., Tokyo, Japan), birds were euthanized, and the isthmus, uterus, and vagina of the laying hens (6 hours after oviposition), molting hens, estrogen-treated molting hens, and control hens (N = 5 per group) were collected.

### 2.2.1. Quantitative RT-PCR analysis for expression of claudins, LITAF, and IFNγ

Quantitative reverse transcription (RT) polymerase chain reaction (PCR) analysis was performed as described [16]. Briefly, total RNA was extracted from the mucosal tissues of the isthmus, uterus, and vagina of each bird using Sepasol RNA I Super (Nacalai Tesque Inc., Kyoto, Japan). Extracted total RNA samples were dissolved in TE buffer (10 mM Tris, pH 8.0, with 1 mM EDTA), treated with 1 U of RNase-free DNase (Promega Co., Madison, WI, USA), and put in a PTC-100 programmable thermal controller (MJ Research Inc., Waltham, MA, USA), programmed at 37 °C for 45 minutes and 65 °C for 10 minutes. Concentration of RNA in each sample was measured using Gene Quant Pro (Amersham Pharmacia Biotech, Cambridge, UK).

The RNA samples were reverse-transcribed using ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan), according to the manufacturer’s instructions. The reaction mixture (10 µL) consisted of 1 µg of total RNA, 1 × RT buffer, 1 mM dNTP mixture, 20 U RNase inhibitor, 0.5 µg of oligo(dT)20 primer, and 50 U ReverTra Ace. Reverse transcription was performed at 42 °C for 30 minutes, followed by heat inactivation for 5 minutes at 99 °C using the PTC-100 Programmable Thermal Controller (MJ Research Inc.).

Primers for claudins, LITAF, IFNγ, and RPS17 PCR are shown (Table 1), with PCR performed using Takara Ex Taq (Takara Bio Inc., Shiga, Japan) according to the manufacturer’s protocol. The PCR mixture (25 µL) contained 0.5 µL of cDNA, 1 × PCR buffer, 1.5 mM MgCl2, 0.2 mM each dNTP, 20 U Taq polymerase, and 0.5 µM each primer. Amplification was performed in a PTC-100 Programmable Thermal Controller (MJ Research Inc.) under the following conditions: 94 °C for 30 seconds, and then 33 cycles at 95 °C for 30 seconds for denaturing, 65 °C for 30 seconds for annealing, and 72 °C for 45 seconds for extension. Thereafter, PCR products were separated by electrophoresis on a 2% (wt/vol) agarose gel containing 0.4% (wt/vol) ethidium bromide.

Real-time PCR was performed using a Roche Light Cycler system (Roche Applied Science, Indianapolis, IN, USA). The

### Table 1

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Sequences 5′–3′</th>
<th>Accession number</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Claudin-1</td>
<td>F: GAC TCG CTC CTT AAG CTG GA&lt;br&gt;R: AAA TCT GGT GTC AAG GGC TG</td>
<td>AY750897</td>
<td>[10]</td>
</tr>
<tr>
<td>Claudin-3</td>
<td>F: AGC CCTCCA TCT CAG CAG&lt;br&gt;R: TTC TCC GGC ACA TCC TCC</td>
<td>NM_204202</td>
<td>[11]</td>
</tr>
<tr>
<td>LITAF</td>
<td>F: TGT GTA TGT GCA GCA ACC CGT ACT&lt;br&gt;R: GCC ATT GCA ATT TGG ACA GAA GT</td>
<td>AY765397</td>
<td>[18]</td>
</tr>
<tr>
<td>IFNγ</td>
<td>F: ACT GAG CCA GAT TGT TTC GAT GT&lt;br&gt;R: TGC TAG TAG CAA TGG CAT CCT CT</td>
<td>X99774</td>
<td>[19]</td>
</tr>
<tr>
<td>RPS17</td>
<td>F: AAG CTG CAG GAG GAG GAG AGG&lt;br&gt;R: GGT TGG ACA GGC TGC CCA AGT</td>
<td>NM_204217</td>
<td>[16]</td>
</tr>
</tbody>
</table>

Abbreviations: F: forward; R: reverse.
reaction mixture (20 μL) contained 3 μL of cDNA, 1× SYBR Premix Ex Taq (Takara Bio Inc.), and 0.5 μM each primer. The mixture was placed into 20 μL capillaries (Roche Diagnostics GmbH, Mannheim, Germany). The cycle parameters for PCR reaction were 95 °C for 5 seconds and 62 °C for 20 seconds. Real-time PCR data were analyzed by the 2^ΔΔCT method to calculate the relative level of mRNA in each sample using RPS17 for the housekeeping gene [20]. Samples of each oviductal segment of a laying hen were used as standard samples for analysis of differences in the expression between laying and molting hens, and samples from a nontreated molting hen were used as standard for comparison between EB-injected and control hens. Results were expressed as relative expression obtained from the ratio between the experimental samples and the standard sample.

2.2.2. Claudin-1 immunohistochemistry

Tissue samples of isthmus, uterus, and vagina from each bird were fixed in 10% (vol/vol) formalin–PBS, dehydrated, and embedded in paraffin. Their sections (4 μm in thickness) were air-dried on slides, and used for hematoxylin and eosin staining and immunohistochemistry for claudin-1. For immunohistochemistry, sections were deparaffinized and autoclaved in 10 mM sodium citrate, pH 6.0, for 1 minute. After washing in PBS (three times for 5 minutes each), the sections were incubated with 5% (vol/vol) normal horse serum for 30 minutes, followed by overnight incubation with goat anti-claudin-1 polyclonal antibody (Santa Cruz Biotech., Inc., Santa Cruz, CA, USA) diluted at 1:400 in PBS. After washing in PBS, sections were incubated with biotinylated donkey anti-goat IgG (Santa Cruz Biotech., Inc.) for 1 hour. The immunoreaction products were detected by incubating with avidin-biotin-peroxidase complex (Vector Lab. Inc., Burlingame, CA, USA) for 1 hour, followed by color development using a reaction mixture of 0.02% (wt/vol) 3′,3′-diaminobenzidine-tetrahydrochloride and 0.005% (vol/vol) H2O2 in 0.05 M Tris–HCl, pH 7.6 (DAB–H2O2 mixture). Normal goat IgG was used for control staining instead of the first antibody. Sections were counterstained with hematoxylin, dehydrated with a graded series of alcohols, and covered.

2.3. Evaluation of mucosal barrier function

Barrier function of mucosal epithelium in the uterus was evaluated by influx of FITC-dextran in the epithelial tissue. Laying and molting hens and molting hens of the EB group were used (N = 5 each). The uterus was exposed surgically under anesthesia (Somnopentyl; Kyoritsu Seiyaku Inc.). An FITC-dextran solution (5 mL per bird) was injected using a syringe with a 23 ga needle into the uterus. The solution was prepared by dissolving FITC-dextran (3000 to 5000 molecular weight; Sigma-Aldrich Co.) in PBS at a concentration of 250 μM. The abdominal wall was closed and birds were allowed to recover. Because invasion of FITC-dextran occurred in mouse intestinal mucosa by 3 hours [21], at 3 hours postinjection, birds were again given Somnopentyl, and euthanized. Uterine tissue was recovered, embedded in cryoembedding medium (Tissue-Tek O.C.T. Compound; Sakura Finetek Inc., Torrance, CA, USA), and snap-frozen in a mixture of dry ice and isopentane. Cryostat sections were air-dried on slides, fixed with 10% (vol/vol) formalin in PBS, and covered with glycerol. The sections were examined under a fluorescence microscope (Eclipse E600, Nikon, Tokyo, Japan) at a wavelength of 520 nm with a Nomarsky filter. The isthmus and vagina were not examined because the spreading of FITC-dextran solution injected into the uterus was not uniformly observed in these segments. Uterine tissue of noninjected molting hens was also similarly processed to determine the specificity of FITC fluorescence.

2.4. Statistical analysis

Relative expressions of claudins, LITAF, and IFNγ were expressed as mean ± SEM, and the significance of their differences between the laying and molting groups as well as between EB and control groups was examined by Student t test. Differences were considered significant at P < 0.05.

3. Results

Expression of claudin-1, -3, and -5 in the vagina, uterus, and isthmus of laying and molting hens are shown (Fig. 1). The PCR products of the three claudins were identified in each segment of all birds. The relative expression levels of claudin-1 in the vagina, uterus, and isthmus were significantly reduced by approximately less than 0.3-fold in molting hens compared with laying hens within each corresponding segment. The expression level of claudin-3 was significantly reduced in molting hens compared with laying hens; namely, the levels of claudin-3 expression in the three segments were approximately 0.9 to 1.2 in laying hens, whereas they were 0.2 to 0.4 in molting hens. The expression levels of claudin-5 of the three oviductal segments were also significantly declined; namely, approximately 0.9 to 1.1 in laying hens to 0.2 to 0.3 in molting hens.

Expression levels of claudin-1, -3, and -5 in the vagina, uterus, and isthmus of molting hens in control and EB groups are shown (Fig. 2). The expression level was significantly greater in the EB group than in the control group, approximately 15- to 20-fold for claudin-1, 10- to 30-fold for claudin-3, and 7- to 12-fold for claudin-5.

Expression of LITAF and IFNγ in the vagina, uterus, and isthmus of laying and molting hens are shown (Fig. 3). The relative expression levels of LITAF and IFNγ were significantly higher in molting hens than in laying hens within each corresponding segment (Fig. 3).

Localization of immunoreactive claudin-1 in the vagina, uterus, and isthmus are shown (Fig. 4). The mucosal surface of the vagina, uterus, and isthmus was lined by ciliated pseudostratified epithelium in laying (Fig. 4A–C) and molting hens (Fig. 4D–F). Tubular glands were well developed in the lamina propria of uterus and isthmus (Fig. 4B and C). The surface epithelium of each segment was thinner and tubular glands in the uterus and isthmus were involuted in molting hens (Fig. 4D–F). In the molting hens of the EB group, the surface epithelium became thicker than that in the control group (Fig. 4G–I for control group; Fig. 4J–L
for EB group), and tubular glands appeared in the lamina propria of the uterus and isthmus (Fig. 4K and L). In laying hens, the claudin-1 immunoreaction products were identified on the apical and lateral region of the mucosal epithelial cells in each segment (Fig. 4A–C). In molting hens, granule-like immunoreaction products were observed in the mucosal epithelium of the isthmus (Fig. 4F), whereas the products were negligible in the vagina and uterus (Fig. 4D and E). In molting hens of the EB group, immunoreaction products were identified in the lateral region of the mucosal epithelium in the vagina (Fig. 4G), and the apical region of mucosal epithelium in the uterus and isthmus (Fig. 4H and I). In contrast, in the molting hens of the control group, granule-like immunoreaction products were observed in the epithelium of the isthmus (Fig. 4L), but they were negligible in those of the vagina and uterus (Fig. 4J and K).

Mucosal infiltration of FITC-dextran in the uterus of laying hen, molting hen, and molting hen of the EB group are shown (Fig. 5). A fluorescence signal was identified in the apical region of mucosal epithelium in molting hens (Fig. 5B), whereas it was negligible in the laying hens (Fig. 5A) and the EB group (Fig. 5C). Autofluorescence of endogenous substances appeared in the lamina propria of molting hens and EB group hens (Fig. 5B and C); it was also observed in the uterus of molting hens that were not injected with FITC-dextran (Fig. 5D).
of paracellular permeability might be disrupted in molting hens. That the FITC-dextran injected into the uterus lumen invaded the paracellular space of mucosal epithelium in molting hens but not in laying hens, we inferred the barrier function of mucosal epithelium was weakened in molting hens. Thus, it is likely that claudin-1, -3, and -5 expression decreased in association with increased permeability in the regressed oviduct of molting hens. It might allow penetration and colonization of bacteria and associated virulence factors into the mucosal tissue via the paracellular pathway between epithelial cells. Frequency of contamination by Salmonella organisms was higher in the eggs laid by postmolting hens than in those from other phases [12,24,25]. This might be not only because of decline of immune functions, but also to colonization of Salmonella bacteria in the oviductal mucosa of molting hens, in which mucosal barrier function was disrupted.

Expression of LITAF and IFNγ genes was higher in molting hens than in laying hens. Expression of proinflammatory cytokines such as IL1β, IL6, and IFNγ was higher in the oviduct of molting hens than in laying hens [26,27]. The level of LITAF, one of the transcription factors regulating the expression of TNFα, was also higher in the oviduct of molting hens [28]. In previous studies, TNFα and IFNγ disrupted epithelial barrier function by influencing tight junctional functions [13–15,29]. These proinflammatory cytokines downregulated claudin-1 in the salivary epithelium [13]. Thus, proinflammatory cytokines, IFNγ and TNFα-like molecule, might participate in the reduction of claudin expression that increases paracellular permeability of epithelium in the regressed oviduct of molting hens.

In molting birds, exogenous estradiol-17β upregulated expression of claudin-1, -3, and -5 mRNA and claudin-1 protein in the mucosal epithelial cells. However, estradiol-17β had no significant effect on the expression of claudin-4, zona-occludens-1, or E-cadherin, although it modulated expression of occludin in the human cervical epithelial cells [30,31]. In contrast, treatment of murine endothelial cells expressing estrogen receptors with estradiol-17β increased transendothelial electric resistance and upregulation of claudin-5 [32]. Thus, the effects of estrogen on the expression of claudins might differ among different claudins and cell types. In the hen oviduct, it is likely that estrogen upregulated claudin-1, -3, and -5 expression. This might result in enhancement of the epithelial barrier because permeability of FITC-dextran in the uterus mucosal epithelium was reduced.

Claudin-1 and -2 are expressed in spermatocytes and Sertoli cells to form the blood–testis barrier in pheasant testes. The expression of claudin-11, but not claudin-1, was increased by testosterone in testicular organ culture or Sertoli cell primary culture [10]. Expression of claudin-1 and -3 was also identified in the developing embryo of chicks [33,34]; in that regard, Ozden et al. [11] localized claudin-3, -5, and -16 proteins in the intestinal epithelium during the third week of chick embryonic development. They suggested that, in addition to the barrier and fence functions within the TJ, these claudins might have roles in the differentiation and/or physiological functions of chick intestines. Blanchard et al. [35,36] reported that claudin-1, -3, and -4 were differentially expressed in the mouse

4. Discussion

This was apparently the first report that the expression of claudins, molecules that form TJs, was affected by the egg-laying phase and estrogen in the lower oviductal segments (vagina, uterus, and isthmus) in hens. Important findings were: (1) expression of claudin-1, -3, and -5 genes and claudin-1 protein in the lower oviductal segments was higher in laying hens than in molting hens; (2) their expression was upregulated by estradiol; (3) expression of LITAF and IFNγ genes was higher in molting hens than in laying hens; and (4) more FITC-dextran infiltrated into the intercellular space of the uterus mucosal epithelium in molting hens than in laying hens and estradiol-treated molting hens.

Tight junctions create the variable barrier regulating paracellular movement of molecules through the epithelium and maintain tissue homeostasis [8,22,23]. Claudins are a family of TJ membrane proteins expressed in various epithelial tissues [8]. Based on gene expression of claudin-1, -3, and -5, we inferred that a TJ was formed in the mucosa of the lower oviductal segments in hens. In addition, immunohistochemistry confirmed the presence of claudin-1 in the mucosal epithelium of these oviductal segments.

In the current study, expression of claudin-1, -3, and -5 mRNA and immunoreactive claudin-1 were reduced in the oviductal mucosa of molting hens. Perhaps these TJs function act as a barrier to invading agents and regulation
Fig. 4. Localization of immunoreactive claudin-1 in the vagina, uterus, and isthmus of laying and molting hens with or without estradiol benzoate injection. (A–C) Laying hens. Immunoreaction products were localized in the apical (short arrows) and lateral region (long arrows) of the mucosal epithelial cells in the three segments. (D–F) Molting hens. Immunoreaction products were negligible in the vagina and uterus (D and E), whereas granule-like products were identified in the mucosal epithelium of the isthmus (F, arrows). (G–I) Molting hens injected with estradiol benzoate. Immunoreaction products were identified in the apical (short arrows) and lateral regions (long arrows) of the mucosal epithelium in each segment. (J–L) Molting hen injected with sesame oil. Immunoreaction products were identified only in the isthmus (L, arrows). Asterisks indicate tubular glands. Scale bars = 50 μm. E, mucosal epithelium; L, lumen of oviduct; Lp, lamina propria.
mammary gland during pregnancy, lactation, and involution, suggesting that different claudin family members might have functional importance at different times during mammary gland development. Thus, claudins might be expressed in various developing tissues to regulate their morphogenesis, growth, and differentiation [8,9]. Estrogen upregulated the expression of claudin-1, -3, and -5 in the lower oviductal segments (Fig. 2) in association with the development of these tissues. The surface epithelium also became thicker in estrogen-treated molting hens in this study. The expressed claudins might play roles not only in barrier formation, but also in development and differentiation of oviductal mucosal epithelium.

4.1. Conclusions

Claudin-1, -3, and -5 were expressed in mucosal tissue of the lower oviductal segment in laying hens. Their expression declined in molting hens, but was upregulated by estrogen. The barrier functions of the mucosal epithelium might be disrupted because of reduced claudin expression, which might increase susceptibility of mucosal tissue during the molting phase.

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