Doctoral Thesis

Studies on the mucosal barrier system in the oviduct of hens

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Hiroshima University

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LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AP1: activated protein 1

BW: body weight

cDNA: complementary deoxyribonucleic acid

DAB: 3,3-diaminobenzidine tetrahydrochloride

dNTP: deoxyribonucleotide tetrahydrochloride

EB: estradiol benzoate

EDTA: ethylene diamine tetraacetic acid

FITC: fluorescein isothiocyanate

H_2O_2: hydrogen peroxide

IFNɣ: Interferon-gamma

IκB: inhibitor of κB

Iκκ: inhihitor of κB kinase

LITAF: lipopolysaccharide-induced TNFα factor

LPS: lipopolysaccharide

MAPK: mitogen-activated protein kinase

mRNA: messenger ribonucleic acid

M: molar

MyD88: Myeloid differentiation factor 88

NFκB: nuclear factor-κB

PBS: phosphate buffered saline

RPS17: ribosomal protein S17

RT-PCR: reverse transcriptase polymerase chain reaction

SEM: standard error of mean

TE: tris-EDTA

TLRs: Toll-like receptors

TNFα: tumor necrosis factor alpha

TRIF: Toll-interleukin 1 receptor domain-containing adaptor-inducing IFN-β

TRAM: TRIF-related adaptor molecule

U: unit

Chapter 1

GENERAL INTRODUCTION

Poultry has a long history to contribute human life providing of large quantities of high quality food in the form of meat and eggs. Their meat and eggs are rich sources of protein, fat, minerals and vitamins for human, and are almost accepted by all people irrespective to their cultures and religions. Almost every country in the world has a poultry industry of some kind, and the production of both layers and broilers is steadily increasing in many countries. As the growth of population, the availability of land for crop cultivation and the food supply is becoming insufficient. In order to mitigate the increasing demand of food, boosting of poultry production is one of the preferential options as it does not require large amount of land as well as much investment in comparison to other agricultural activities (Panda, 1989).

Diseases in poultry

Poultry diseases have been an important limiting factor to profitable poultry production. The agents that cause disease fall into five groups, namely viruses, bacteria, fungi, protozoa, and helminth (worms). Diseases happen when exposure combined with the virulence of an organism is greater than resistance of the host. Among those diseases, fowl typhoid caused by *Salmonella (S) enterica* serovar *gallinarum* and serovar *pullorum* is a common source of poultry diseases (Chappell et al., 2008). Fowl typhoid caused by *S. gallinarum* is characterized by anaemia, hepatospelnomegaly, and haemorrhage of the intestinal tract in the latter stages (Shivaprasad, 2000). It has been reported that *S. pullorum* and *S. gallinarum* (Hossain et al., 2006), *S. enteritidis* (Keller et al., 1995), *Mycobacterium, Mycoplasma gallisepticum, E. coli, E. intermedia, Proteus* spp., *Staphylococcus,* and *Yersinia pseudotuberculosis* (Sharma and Singh, 1968; Keymer 1980; Martin del las Mulas et al., 1990) can invade the avian female reproductive system including ovary and oviduct. Wigley et al. (2001) reported that fowl typhoid caused by *S. pullorum* resulted in significant mortality in chicks, but it was less frequent in birds of more than a-week-old

chicks. On the other hand, Jones et al. (2001) reported that infection with *S. gallinarum* was responsible of a mortality rate of 60% in 3-week-old outbred chickens. Not only bacteria infecting birds, but also many viruses such as infectious bronchitis and avian influenza virus are able to invade birds (Hofstad et al., 1978; Takata et al., 2003; Capua and Alexander, 2004; De Buck et al., 2004a, b; Promkuntod et al., 2006; Chousalkar and Roberts, 2007). Infection by them causes significant economic losses due to increased chick mortality in the poultry industry worldwide.

The responses against infection by potential pathogenic microorganism are known as immune response. Immune response of the birds can be built by vaccination. Some poultry diseases are more economically or effectively controlled by vaccination such as Newcastle disease, Marek's disease, fowl pox, infectious bronchitis, and infectious bursal disease. Contamination by workers, visitors, birds, feed, and equipments in the poultry farm have to be avoided. Drugs and medications assist to fight the disease organisms after they have overwhelmed the bird's natural defenses (http://www.answers.com/topic/poultry-farming).

Since the oviduct is the organ responsible for the reproduction, and it is a susceptible site for invasion with infective microorganisms mentioned above, the immune system of oviduct is an important concern to the poultry industry. Deviation or alteration in the oviduct function caused by microorganism infection can directly results in a production of contaminated eggs, low quality eggs, and abnormal eggshell as well as unhealthy chicks (Wigley et al., 2001; Chousalkar and Roberts, 2007). Microbial infection in the oviducts can cause several reproductive disorders such as oophoropathies and oviduct obstruction (Keymer, 1980), salpinoperitonitis (Lindgren, 1964), and salpingitis (Domermuth et al., 1967; Sharma and Singh 1968). Therefore, understanding of the role of the immune system in the protection of the oviduct is a crucial point to successful reproduction as well as preventing pathogens to enter the egg, resulting in the production of hygienic eggs and healthy chicks.

The hen reproductive organs

The reproductive organs including ovary and oviduct develop only in the left side of body in hens. The right reproductive organs regress during embryo phase. Ovarian functions are regulated by anterior pituitary secretes gonadotropins, namely

follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Oviducts growth, differentiation and functions are regulated by gonadal steroids including estrogen (Johnson, 2000; Bahr, 1991). Figure 1 shows oviducts of laying, molting, and molting then with oil or estradiol benzoate.

The ovary

The ovary consists of the medulla and cortex containing numerous small follicles, five to ten preovulatory follicles, and several postovulatory follicles in improved chickens. The preovulatory follicles grow rapidly, and are arranged in hierarchy based on their size. They are idenfied according to size with the largest follicle (F1) destined to ovulate, and the second largest follicle (F2) to ovulate next day, and so forth. After ovulation of F1, less mature follicles move up to one position in hierarchy. Unlike in mammals corpus luteum is not formed. One follicle ovulates in one day with an ovulation cycle of 24-26 h. The follicle consists of oocyte surrounded by oocyte plasma membrane, perivitelline layer, granulosa layer and theca layers. In immature small yellow follicle, the granulosa layer consists of several layers of cuboidal cells. With growing in size, these cells become stretched and changed to a single flattened layer. These cells in the largest follicle produce progesterone in response to circulating luteinizing hormone (LH). The theca tissue is a fibrous connective tissue, containing nerves, blood vessels and other specialized theca cells that secrete androgen and estrogen (Johnson, 2000; Bahr, 1991). Secretion of FSH and LH are regulated by gonadotropin releasing hormone from hypothalamus as well as ovarian sex steroids and inhibins (Vanmontfort et al., 1992).

The oviduct

The oviduct of mature laying hen consists of infundibulum, magnum, isthmus, uterus, utero-vaginal junction, and vagina. The left oviduct is derived from the left Müllerian duct. The left oviduct develops after 16 weeks of age and becomes fully functional just prior to the onset of egg production under the control of gonadal steroids. Numerous leaflet or pleated folds (plicae) are observed in the luminal mucosa of the oviduct. Among different segment of the oviduct, these folds vary in height, coloration

and density (Bakst, 1987). The mucosal cells covering the luminal folds consist of two types of epithelial cells, non-ciliated and ciliated cells. Non-ciliated cells are secretory in function and contain secretory granules in their cytoplasm. On the other hand, the ciliated cells may or may not contain secretory granules (Makita et al., 1973). The mucosal tissues of magnum, isthmus, and uterus contain tubular glands which secrete albumin, shell membrane, and shell components, respectively. Sperm storage tubules which preserve spermatozoa are localized in the infundibulum and utero-vaginal junction (Fujii and Tamura, 1963; Aitken, 1971; Kami and Yasuda, 1984; Yamamoto et al., 1985; Bakst, 1987).

Infundibulum: Infundibulum can be subdivided into the funnel and the neck. The funnel consists of a thin-walled, funnel-shaped opening, flattened in the dorso-ventral direction with its flared lips lying in close proximity to the ovary. The funnel-walls converge rapidly to form the infundibular neck, a narrow thin-walled tube which rapidly increases in size and thickness to form the magnum. The lips and wall of the funnel are lined with a uniform ciliated epithelium. Infundibulum functions to engulf the ovum after ovulation and secrets components of the outer vitelline membrane, the chalaza, and the first layer of albumen to the ovulated ovum. Fertilization also occurs in its tubular part before outer vitelline membrane is formed. The ovulated ovum takes approximately 15-30 min to pass through the infundibulum (Hodges, 1974).

Magnum: Magnum, or albumen-secreting region, is the largest segment of the oviduct. The mucosal ridges are considerably increased in number, height and thickness, the greater part of this development being due to the intense development of the tubular glands. The mucosa is lined by an epithelium consisting of ciliated columnar cells and secretory goblet cells. Tubular glands secrete albumen to the passing eggs, whereas it has less-developed muscular layer (Hodges, 1974). Secretion of albumin in magnum may be regulated by three different factors, namely direct mechanical stimulation by the ovum during it passes through the magnum, a hormonal stimulation, and a neural coordinating stimulation (Gilbert, 1971). Estrogen stimulates the epithelial stem cells to develop into three morphologically different cell types: (1) tubular gland cells responsible for ovoalbumin, lysozyme and conalbumin secretion, (2) ciliated cells, and (3) goblet cells which synthesize avidin following exposure to progesterone and estrogen (Tuohimaa et al., 1989). The ovum passes through the entire length of magnum within 2-3 h.

Isthmus: The boundary between the magnum and isthmus is clearly delineated by a narrow, translucent zone in the oviduct which contains no tubular glands. The surface epithelium is tall, and consists of alternating ciliated and secretory (glandular) cells. The ciliated cells retain their columnar form with an apical nucleus throughout the secretory cycle of the isthmus, being un-constricted by adjacent glandular cells. Development of goblet cell structure occurs but they never become typical goblet cells. The tubular gland cells of the isthmus do not undergo distinct secretory phases such as are found in the magnum, although there are some differences between the phases of secretory activity and rest. When the isthmus is secreting the shell membranes, the gland epithelium shows maximum activity. Each epithelial cell becomes full of spherical, deeply-staining granules of variable size. The egg passes through the isthmus in approximately 1 to 2 h, and the inner and outer egg-shell membranes are formed during this period (Hodges, 1974).

Uterus: Uterus is called the shell gland which is characterized by prominent longitudinal muscle layer lined internally with mucosal tissue including tubular gland, whereas the luminal margin is lined with the mucosal surface epithelium. The blood supply to the uterus has been considered in greater detail than that for any other part of the oviduct. Macroscopically, the uterus is divided into two parts; the first is the cranial tube-shaped portion (red region or tubular uterus) and the second is the main part (pouch). The surface epithelium, which is approximately 30 μm in height, consist of a single layer of columnar cells with alternating apical and basal nuclei (Hodges, 1974). During the first 8 hours in the uterus, plumping of the eggs occurs, which is a process of adding more water to the egg so that the egg weight becomes doubled after this period. Calcification of the egg initiates in the red region where the mammillary cores are formed and the first deposition of calcium carbonate takes place (Johnston et al., 1963; Simkiss, 1968; Wyburn et al., 1973; Solomon, 1975; Stemberger et al., 1977), and then subsequent calcification occurs in the pouch to complete the eggshell formation. In some species that lay pigmented eggs, shell pigments are deposited by the ciliated cells. The ovum is kept in the uterus for approximately 20 h (Johnson, 2000).

Vagina: Vagina is a short and narrow muscular duct. This part is the caudal-most segment of the oviduct and opens to the cloaca. It is separated from the pouch uterus by the uterovaginal junction (UVJ) that contains sphincter muscle and sperm

storage tubules. The mucosa of the vagina is raised into numerous, narrow, low longitudinal ridges. There is no layer of tubular glands within the corium of the folds and thus the surface epithelium is thrown into many secondary folds. The surface epithelium of the mucosal folds consists of alternating ciliated cells, with apical nuclei, and non-ciliated, mucous-secreting glandular cells, possessing basal nuclei. The height of the epithelium, especially over the crests of the folds, is greater than that found in the uterus. The vagina may not have role in the process of egg formation but it secrets the cuticle layer to the outer surface of the eggshell prior to oviposition and coordinates with the shell gland in the expulsion of the egg for oviposition (Hodges, 1974).

Role of estrogen on the oviduct

Estrogen drives the proliferation and differentiation of several cell types, regulates reproductive behavior and protects from apoptosis of cells in reproductive organs (Dougherty and Sanders, 2005). Munro and Kosin (1943) showed that injection of estrogen into sexually immature chicks stimulated massive growth of the oviduct. Estrogen is also responsible for directing cytodifferentiation. In the oviduct, epithelial cell differentiate into tubular gland cells, goblet cells and ciliated cells. Not only does estrogen trigger cell proliferation and differentiation in the chick oviduct, but also protects against cell death. Moreover, withdrawal of estrogen causes a linear increase in apoptosis during the first few days in vivo (Oka and Schimke, 1969; Dougherty and Sanders, 2005).

Immune system

The immune system functions to provide a protection from infectious agents and the damage that they cause, and from other harmful substances such as toxins, by a variety of effector cells and molecules (Abou El Azab et al., 2008). To improve the individual affectivity against disease, the immune system must fulfill four main tasks, namely immunological recognition, immune effector functions, immune regulation, and immunological memory. When an individual first encounters an infectious agent, the initial defenses against infection are physical and chemical barriers that prevent microbes entering the body (Murphy et al., 2007). The primary mechanism of immune

response in chickens resembles to that in mammals, although birds have a bursa of Fabricius where B cells differentiate (Sharma, 1991; Lillehoj and Okumura, 2003). Like in mammals, the immune system in the newly hatched chickens is not fully developed and hence they are susceptible to many pathogens during the first few weeks of age, so that they require initial immunological assistance. Hence, passive maternal immunity is accepted to be an antigen-specific protection through antibodies (Tizard, 2004).

Innate immunity

The response to an initial infection occurs in three phases. These are the innate immunity, the early induced innate response, and the adaptive immune response. The first two phases rely on the recognition pathogens by germline-encoded receptors of the innate immune system, whereas adaptive immunity uses variable antigen-specific receptors that are produced as a result of gene segment rearrangements (Murphy et al., 2007). The recognition of antigens and responding by the innate immune response starts to take action at the moment when the innate immune cells encounter and recognize any harmful antigens. On the other hand, adaptive immune cells takes days to weeks to be fully established. Adaptive immunity occurs late, because the rare B and T cells specific for the invading pathogen must first undergo clonal expansion before they differentiate into effector cells that can clear infection. The effector mechanisms that remove the infectious agent are similar or identical in each phase (Murphy et al., 2007).

The body organs of an organisms is lined externally (skin and the moist surfaces of the eyes) as well as internally (nose, airways and the lungs, mouth and the digestive tract, and the urinary and reproductive systems) with epithelial cells. The epithelial and phagocytic cells are the major components of the innate immune system. This epithelium forms the outer mechanical barrier and produce effector molecules to protect the organism from being invaded with infectious agents. The innate antimicrobial properties of epithelial surfaces were noted a century ago (Ganz, 2003). The internal epithelia are known as mucosal epithelia because they secrete a viscous fluid called mucus, which contain many glycoprotein called mucins. Surface epithelia are more than mere physical barriers to infection; they also produce chemical

substances that are microbicidal or that inhibit microbial growth (Ganz, 2003; Murphy et al., 2007).

Adaptive immune response

An adaptive immune response is induced when an infection overwhelms innate defense mechanisms, which is divided into two classes, T cell-mediated immune responses and humoral immunity. T cells are involved in the cellular immunity, and B cells mediate humoral immunity by secreting antibodies. Cell-mediated immune responses are directed principally at intracellular pathogens. They involve the destruction of infected cells by cytotoxic CD8 T cell, or destruction of intracellular pathogens in macrophages. When birds were exposed to any antigens, the antigen presenting cells (APC) take them and present antigens to T cells with major histocompatibility complex molecules (MHC) on their surface. Antigens presented by MHC class II are recognized by helper/inducer T cells ($CD4^+$ T cells), which induce B cell maturation. Matured B cells produce antibodies, namely three types of immunoglobulins (Ig), IgG, IgM, and IgA. The $CD4^+$ T cells might also enhance the activity of macrophages. In contrast, when an intracellular infection by microorganisms occurs, the infected cells present the internal antigens with MHC class I, and are removed by cytotoxic T cells ($CD8^+$ T cells). MHC class II is expressed by specific APC including macrophages, whereas MHC class I is expressed by all nucleated cells (Sharma, 1991; Murphy et al., 2007).

Toll-like receptors (TLRs)

TLRs were originally discovered in the fruitfly *Drosophilla* as receptors for embryonic development and antifungal molecules (Lemaitre et al., 1996), and later, homologous molecules were discovered in birds and mammals (Yilmaz et al., 2005; Higgs et al., 2006). TLRs are known to recognize one or more microbial molecular patterns, generally by direct interaction with molecules on the pathogen surface. Some TLRs are located on the cell surface of dendritic cells, macrophages and other cells, where they are able to detect extracellular pathogen molecules. TLRs located intracellularly, in the wall of the endosome, can recognize microbial components, such

as DNA, that are accessible only after the microbe has been broken down (Medzhitov, 2001; Takeda and Akira, 2003; Yilmaz et al., 2005).

Expressions of TLRs-1 to -11 are identified in mammals. TLR-1 forms heterodimers with TLR-2 and recognizes Tri-acyl peptides (Ozinsky et al., 2000; Hornung et al., 2002; Hopkins and Sriskandan, 2005). TLR-2 forms heterodimers with TLR-1 and -6 to recognize synthetic triacylated or diacylated lipopeptides (Aliprantis et al., 1999; Takeuchi et al., 2001; 2002). TLR-3 recognizes double-stranded RNA and TLR-4 works with association of CD14 (Hemmi et al., 2002; Dunzendorfer et al., 2004; Ulevitch, 2004) to recognize LPS of Gram-negative bacteria (Hornef et al., 2003; Iqbal et al., 2005). TLR-5 recognizes flagellin while TLR-7 and TLR-8 are stimulated by single stranded RNA of some viruses and some other synthetic compounds (Heil et al., 2003; Philbin et al., 2005; Iqbal et al., 2005). TLR-9 recognizes unmethylated CpG oligo DNA of bacteria and viruses, while TLR-10 ligands are still unknown (Hornung et al., 2002; Brownlie et al., 2009). TLR-11 is stimulated by parasitic protozoan and profillin (Yarovinsky et al., 2005).

In chickens, expression of TLR-1 (type 1 and 2), TLR-2 (type 1 and 2), TLR-3, -4, -5, -15, and -21 are identified (Higgs et al., 2006; Ozoe et al., 2009). TLR-2, -3, -4, -5 recognize same ligands as reported in mammals (Fukui et al., 2001; Kurt-Jones et al., 2004; Iqbal et al., 2005). Chicken TLR-7 is sensitive to single stranded RNA and loxorbine which is a guanosine analogue (Philbin et al., 2005; St Paul et al., 2013). TLR-15 and TLR-21 are chicken specific. TLR-15 recognizes a unique, non-secreted, heat stable component of both Gram-negative and Gram-positive bacteria (Nerren et al., 2010) in addition to secreted virulence-associated fungal and bacterial protease (de Zoete et al., 2011). Whereas chicken TLR-21 and mammalian TLR-9 are homologous and recognize same ligand, CpG oligo DNA (Brownlie et al., 2009).

In mammals, TLRs play an important role in the innate immunity through recognition of their ligands. There are two types of TLR signaling pathways, namely myeloid differentiation factor 88 (MyD88-dependent) and toll-interleukin 1 receptor (TIR)-domain-containing adaptor-inducing interferon β (TRIF-dependent pathways). Activated TLRs signaling initiates with recruitment of TIR-domain-containing adaptor molecules, namely MyD88 and TRIF, which act as important messengers to activate down-stream kinases including mitogen-activated protein kinase (MAPK) and inhihitor of κB kinase (Iκκ) and transcriptional factors, namely nuclear factor-κB (NFκB) and

activated protein 1 (AP1), which produce effecter molecules including cytokines, chemokines, inflammatory enzymes such as iNOS and oxidase, and type 1 interferons (Kawai and Akira, 2010; Kogut et al., 2012).

TLR4 in complex with myeloid differentiation 2 (MD2) engages LPS. Five of the six lipid chains of LPS bind MD2 and the remaining lipid chains associates with TLR4. The formation of a receptor multimer composed of two copies of the TLR4-MD2-LPS complex initially transmits signal for the early-phase antivation of NFκB by recruiting the toll-interleukin 1 domain-containing adaptors (TIRAP) and MyD88-dependent pathway. The TLR4-MD2-LPS complex is then internalized and retained in the endosome, where it triggers signal transduction by recruiting TRIF and TRIF-related adaptor molecule (TRAM), which leads to the activation of IFN regulatory factor 3 (IRF3) and late-phase NFκB for the induction of type I interferon (TRIF-dependent pathway). Both early-and late-phase activation of NFκB is required for the induction of inflammatory cytokines (Barton and Medzhitov, 2002; Subedi et al., 2007; Kawai and Akira, 2010). However, in chickens, TRAM is absence on the chicken genome. Thus, MyD88-dependent pathway may deliver signals to kinases including MAPK and Iκκ to activate transcriptional factors, namely NFκB and AP1 (Kogut et al., 2012). Figure 2 shows representative diagrams describing the TLR signaling pathways in chickens.

Mucosal barrier to infection

Figure 3 shows the mucosal barrier systems formed by mucus gel, epithelial cell junctional structures, and leukocyte activity, play essential roles to prevent infection by pathogenic agents in the mucosal tissue of hen oviducts. The surface of the gastrointestinal, reproductive, respiratory, and urinary tracts, and the surface of the eye are defended by epithelia, which provide a physical barrier between the internal milieu and the external world that contains pathogens. Epithelial cells are held together by tight junctions, which effectively form a seal against the external environment. The junctional complex of epithelial cells is located at the most-apical part of the lateral membrane and consists of three components, namely tight junctions, adherens junctions and desmosomes. Tight junctions are formed by some proteins including claudin, occludin, and tricellulin (Tsukita et al., 2001; Forster, 2008).

Infections occur only when the pathogen can colonize or cross through these barriers. The importance of epithelia in protection against infection is obvious when the barrier is breached. Pathogens normally cross epithelial barriers by binding to molecules on the epithelial surfaces of internal organs, or establish an infection by adhering to and colonizing these surfaces. This specific attachment allows the pathogen to infect the epithelial cell, to damage the epithelium so that it can be crossed, or, in the case of colonizing pathogens, to avoid being dislodged by the flow of air or fluid across the epithelial surface (Murphy et al., 2007; Macia et al., 2012).

The internal epithelia are known as mucosal epithelia because they secrete a viscous fluid called mucus, which contains many glycoproteins called mucins. Microorganisms coated in mucus may be prevented from adhering to the epithelium, and in the mucosal epithelia such as that of the respiratory tract, microorganisms can be expelled in the flow of mucus driven by the beating of epithelial cilia. The surface epithelia are more than mere physical barriers to infection. They also produce antimicrobial substances or that inhibit microbial growth. For example, the secreted saliva contains antibacterial enzymes lysozyme, phospholipase A, and histatins. In the intestinal tract, Paneth cells which are resident in the base of the crypts in the small intestine produce cryptdins or α-defensins. Epithelia of respiratory and urogenital tracts, skin, and tongue produce antimicrobial peptides β-defensins. Antimicrobial peptides such as the defensins are cationic peptides that are thought to kill bacteria by damaging the bacterial cell membrane (Murphy et al., 2007; Linden et al., 2008b).

If a microorganism crosses an epithelial barrier and begins to replicate in the tissues of the host, the most cases it is immediately recognized by the mononuclear phagocytes or macrophages, that reside in these tissues. They are found in especially large numbers in connective tissue, in the submucosal layer of the gastrointestinal tract, in the lung, along certain blood vessels in the liver, where they remove senescent blood cells. The second major family of phagocytes, namely neutrophils or polymorphonuclear neutrophilic leukocytes (PMNs) are short-lived cells that are abundant in the blood but are less in healthy tissue. Both of these phagocytic cells have a key role in innate immunity because they can recognize, ingest, and destroy many pathogens without the aid of an adaptive immune response (Murphy et al., 2007; Macia et al., 2012).

As most microorganisms enter the body through the mucosa, the macrophages located in the submucosal tissues are the first cells to encounter most pathogens, but they are soon reinforced by the recruitment of large numbers of neutrophils to the sites of infection. Macrophages and neutrophils recognize pathogens by means of cell-surface receptors that can discriminate between the surface molecules displayed by pathogens and those of the host. These receptors, namely the macrophage mannose receptor, scavenger receptors, and CD14. The CD14 binds the lipopolysaccharide present on the surface of Gram-negative bacteria and allows it to be recognized by TLR4. In many cases, binding of a pathogen to these cell-surface receptors leads to phagocytosis, followed by the death of the pathogen inside the phagocyte (Murphy et al., 2007; Macia et al., 2012).

Aim of study

The primary function of the immune system is to keep the whole body of an organism and its organs in healthy condition to ensure the maximum and optimum performance. The reproductive system is one of the important systems in the body that enables the organisms to multiply and survive on the earth. Thus, it is of great importance to keep oviduct healthy to get healthy chicks and hygienic eggs. It would be believed that understanding the aspects of the chicken reproductive immune system enables us to have a good idea how to prevent infection and diseases. Mucosal barrier systems formed by mucus gel, epithelial cell junctional structures, and leukocyte activity, play essential role to prevent infection by pathogenic agents in mucosal tissue of hen oviduct. Mucosal epithelial cells joined by tight junctions, together with underlying leukocytes, secrete many defensive compounds into mucosal fluid including mucins to form a physical barrier preventing pathogen invasion (Linden et al., 2008b; Macia et al., 2012). The lower segment of the oviduct is the primary tissue where microorganisms colonizing in the cloaca ascend the oviduct. The hen oviduct could be infected by *Salmonella enteritidis* (Keller et al., 1995; Takata et al., 2003; De Buck et al., 2004a). Previous reports suggested that the oviduct was more susceptible in molting hens than in laying hens because contamination of eggs by *Salmonella* was more frequent after resumption of laying in postmolting hens (Golden et al., 2008). T-cell-mediated immune functions may be declined in the oviductal mucosa in molting hens because its frequency on the mucosal surface layer was reduced (Yoshimura et al.,

1997). Proinflammatory cytokines such as TNFα and IFNɣ downregulated expression of claudins and increased paracellular permeability in the epithelium (Baker et al., 2008; Mazzon and Cuzzocrea, 2008). If the expression of mucins and claudins in the oviductal epithelium declines, mucosal barrier functions may be weakened, leading to increased susceptibility to pathogenic agents. It remains unknown whether the mucosal surface barrier formed by mucin and tight junction in the lower oviductal segments changes during the molting phase, compared with that in laying phase. It is also unknown whether expression of mucins and claudins is regulated by estrogen, although the growth of the oviduct is upregulated by this steroid (Oka and Schimke, 1969). LPS located on the surface of Gram-negative bacteria. If expression of mucin is induced by LPS in the hen oviduct, it is probably mediated by TLR4 and its signal pathways, leading to activation of transcriptional factors. The expression of TLR4 has been identified in hen oviduct (Ozoe et al., 2009). However, it is unknown which signal pathway of TLR, and which transcriptional factors, NFκB or AP1, are responsible for mucin expression by LPS.

The goal of this study was to determine the mechanism by which mucosal barrier mediated by mucins and tight junction is formed in the mucosal epithelium of the oviduct. It was focused on the mechanism by which mucin synthesis for mucosal barrier is regulated by oviductal growth, gonadal steroid and bacterial component, LPS, in the lower segment of oviduct, namely vagina, uterus or isthmus. Then, the existence of the epithelial barrier formed by tight junction was also examined.

In Chapter 1, general introduction including significance of poultry reproductive immunology studies and the objective of this study were described. In Chapter 2, the profiles of mucin expression in the oviduct of laying, molting, and estradiol-treated molting hens were investigated. The protein localization and gene expression of mucin and localization of lectin-reactive sugar substances that may be incorporated into mucin were examined in the lower oviductal segments (vagina and uterus) in laying, molting, and estradiol-treated molting hens.

In Chapter 3, it was examined whether the mucin expression was induced by LPS in oviduct of hens. Effect of LPS on mucin expression in the laying and molting hens, and effect of estrogen in the sensitivity to LPS for mucin induction in the hen oviducts were examined. Then, in Chapter 4, the intracellular signaling molecules in the vaginal mucosa for mucin induction, and the effect of molting and estrogen on their

expression were investigated. Expression of TLR4, its adaptor molecules, and transcriptional factors in the vaginal mucosa of laying and molting hens treated with or without estradiol were examined.

In Chapter 5, the profiles of tight junction molecule expression in the oviduct of laying, molting, and estradiol-treated molting hens were investigated. The protein localization and gene expression of claudin-1, -3, and -5, expression of LITAF and IFNγ, and permeability of fluorescein isothiocyanate (FITC)-dextran within mucosal epithelium in the lower oviductal segments in laying, molting, and estradiol-treated molting hens were examined.

A general discussion of the overall results to characterize mucosal barrier mediated by mucins and claudins in the lower oviductal segments in hen was described in Chapter 6, followed by Chapter 7 which is a brief summary of the overall study.

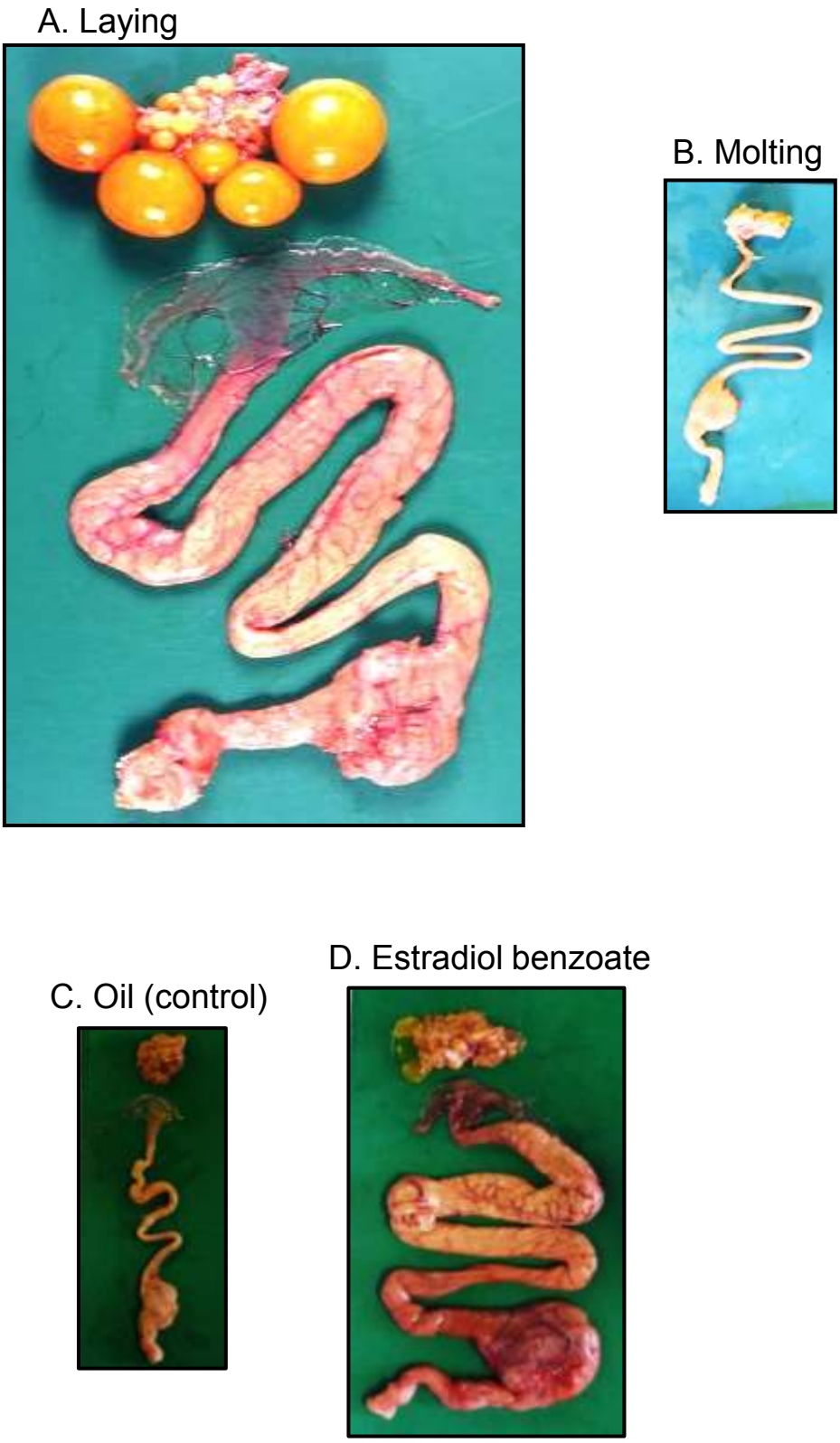

Figure 1. Oviducts of laying and molting hens.
A: Laying hen.
B: Molting given with restricted feed (25g/d for 20d).
C: Molting hen daily injected with sesame oil (100 µL) for 7 days.
D: Molting hen daily injected with estradiol (1 mg) for 7 days.

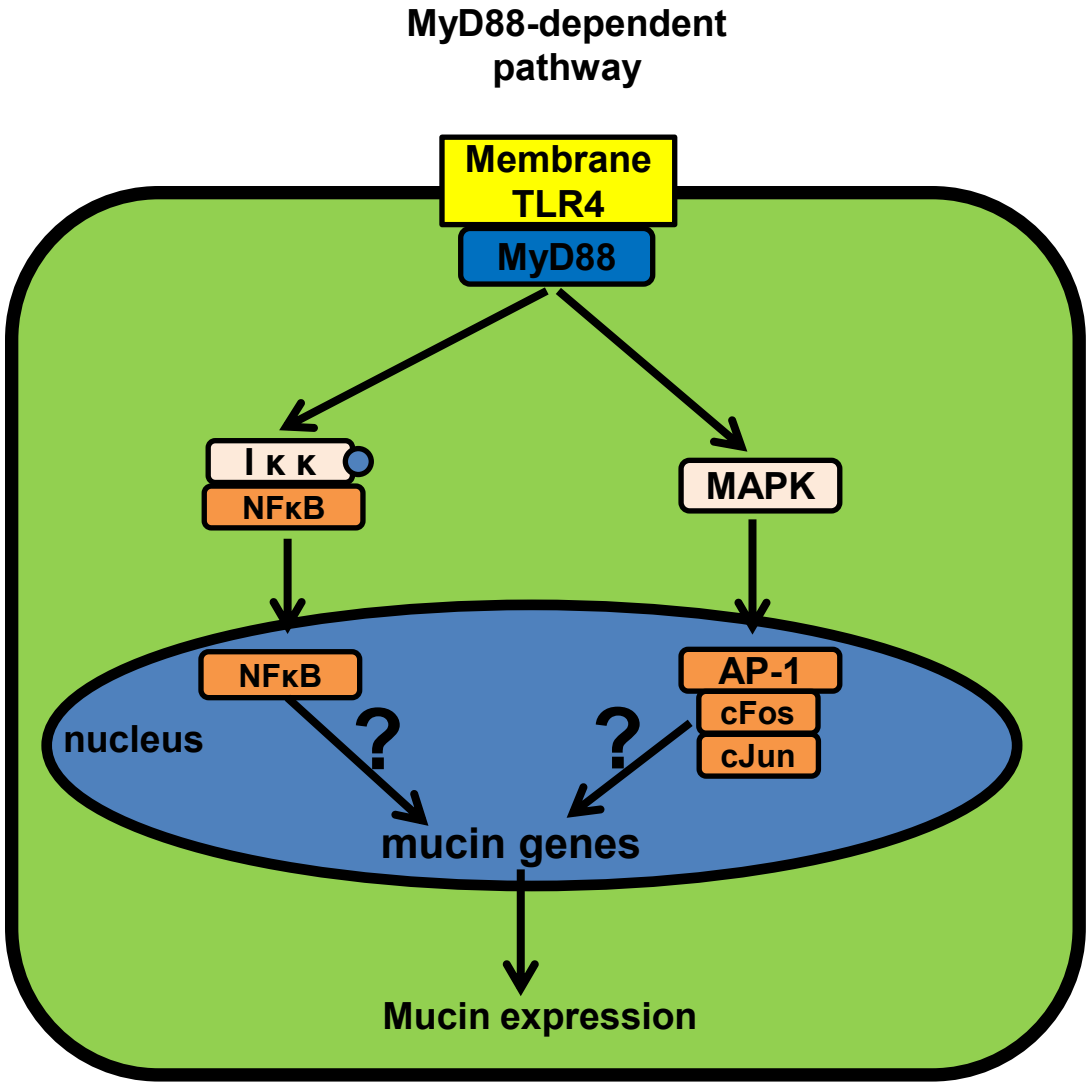

Figure 2. Representative diagrams describing the toll-like receptor (TLR) signaling pathways in chickens. Myeloid differentiation factor 88 (MyD88)-dependent pathway may delivers signals to kinases including mitogen-activated protein kinase (MAPK) and inhihitor of κB kinase (Iκκ) to activate transcriptional factors, namely nuclear factor-κB (NFκB) and activated protein 1 (AP1).

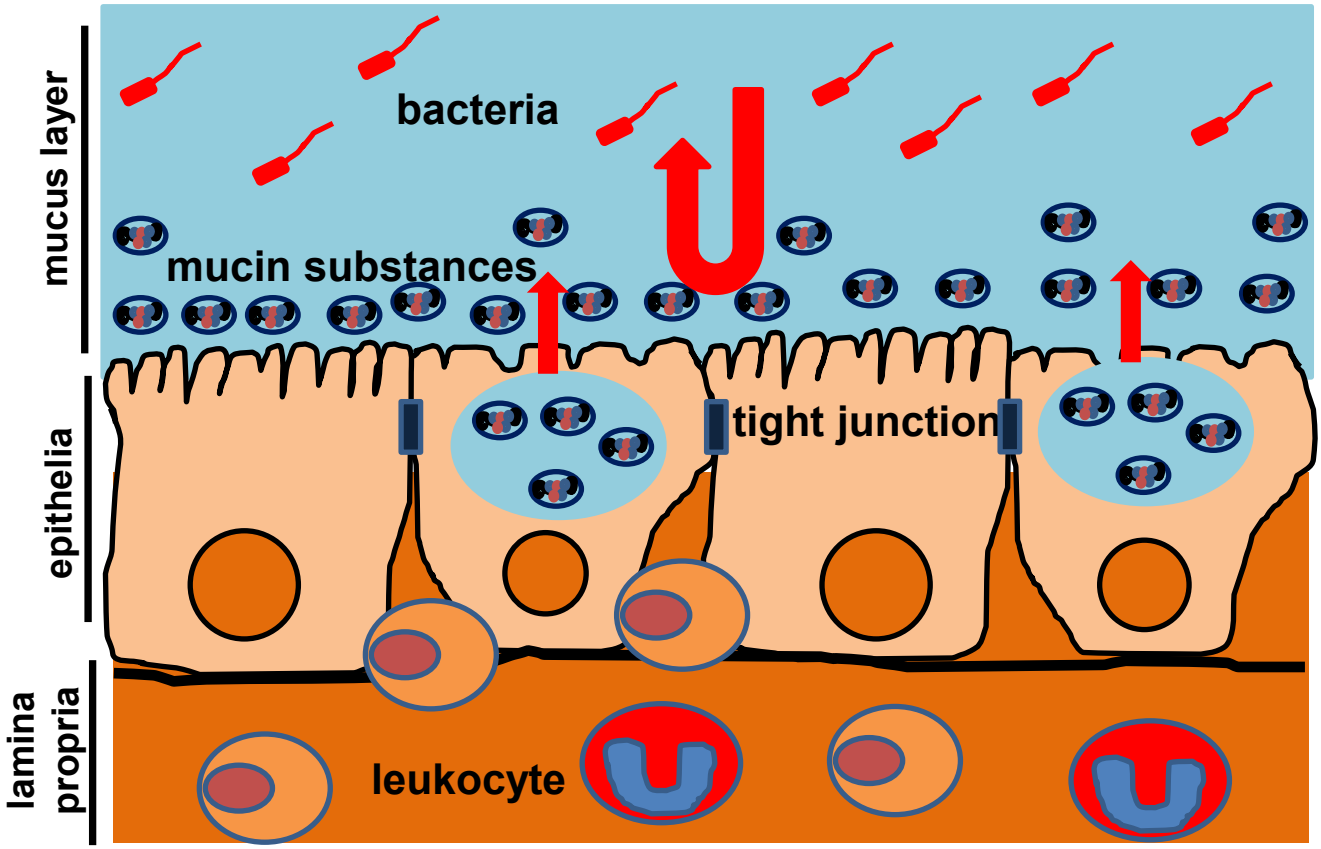

Figure 3. Representative diagrams describing the mucosal surface structure and its components. The mucosal barrier is formed by mucus gel, epithelial cell, junctional structures, and leukocyte activity.

Chapter 2

FORMATION OF MUCOSAL SURFACE BARRIER BY MUCIN IN THE LOWER OVIDUCTAL SEGMENTS AND ITS CHANGES WITH EGG-LAYING PHASE AND GONADAL STEROID STIMULATION IN HENS

INTRODUCTION

The mucosal tissue of the oviduct fulfills defense functions that are essential for maintaining its health. Mucosal epithelial cells form a contagious lining that acts as a barrier against the moist exterior environment. The surface of the epithelial cell lining is covered by a mucus layer which protects the underlying epithelium from pathogenic microorganisms (Corfield et al., 2000; Perez-Vilar, 2007; Linden et al., 2008a). Mucins are composed of glycoproteins and secreted by mucosal epithelium (Gendler and Spicer, 1995; Linden et al., 2008a). They can be divided into three distinct subfamilies: (1) secreted gel-forming mucins, (2) cell-surface mucins, and (3) secreted non-gel-forming mucins. Gel-forming mucins, such as mucin5AC, are the major constituent of mucus and confer its viscoelastic properties, and cell surface mucins are prominent feature of the apical glycocalyx of the mucosal epithelia (Linden et al., 2008a). Sugar residues may be incorporated into the glycoprotein of mucin and also be responsible for the attachment of parasites and microbes to the mucosal epithelium (Van Poucke et al., 2010).

Sugar residues could be identified and characterized by lectins. Lectins bind to a specific sugar residue of glycoprotein with high affinity. WGA, a lectin from wheat germ agglutinin (*Triticum vulgaris*), binds specifically to N-acetylglucosamine (GlcNAc) and N-acetylneuraminic acid (sialic acid). Jacalin lectin, the major protein from jackfruit (*Artocarpus heterophylus*) seeds, shows highly specific binding to galactose (Gal) and N-acetylgalactosamine (GalNAc) (Kabir, 1998; Tatsuzuki et al., 2009; Fallis et al., 2010). Jung et al. (2011) reported that the epithelium and tubular gland cells of hen

oviductal magnum were positive for WGA lectin, suggesting that these cells contained GlcNAc and sialic acid residues.

Mucin5AC, a secreted gel-forming mucin, was reported to be expressed in various tissues, including the respiratory tract, stomach and cervix in mammals (Porchet et al., 1995; Gipson, 1997; Buisine et al., 1998; Bara et al., 2003). Previous studies in chicks showed increased mucin mRNA expression in the intestine by starvation (Smirnov et al., 2004), probiotic feeding (Smirnov et al., 2005), and in ovo administration of carbohydrates (Smirnov et al., 2006). Immunoreactive mucins including mucin5AC in the distal colonic epithelium of mice were reported to be decreased by infection with *Citrobacter rodentium* (Linden et al., 2008b). Thus, mucin synthesis may be affected by physiological and environmental factors in mucosal tissues.

Lower segment of the oviduct is the primary tissue where microorganisms colonizing in the cloaca ascend the oviduct. The hen oviduct may be more susceptible to pathogens during the molting than laying phase because contamination of eggs by *Salmonella* organisms was identified more frequently just after resumption of egg laying after molting (Keller et al., 1995; Holt, 2003). T-cell-mediated immune functions may be declined in the oviductal mucosa in molting hens because its frequency on the mucosal surface layer was reduced (Yoshimura et al., 1997). However, it remains unknown whether the mucosal surface barrier formed by mucin in the oviduct changes during the molting phase compared with that in the laying phase. It is also unknown whether expression of mucin is regulated by estrogen, although the growth of oviduct is up-regulated by this steroid (Oka and Schimke, 1969).

Thus, the goal of this study was to determine differences in the mucin expression that forms the mucosal surface barrier in the lower oviductal segments (vagina, uterus and isthmus) at different egg-laying phases, namely, laying and molting, and the role of gonadal steroid in their expression in hens. Sugar residues of mucin protect mucosal tissue from proteolitics enzymes of mucosal pathogens (Linden et al., 2008b). The specific questions were whether: (1) expression of mucin, (2) localization of WGA lectin- and Jacalin lectin-positive substances in the lower oviductal segments were different between laying and molting and changed by estradiol benzoate (EB) stimulation.

MATERIALS AND METHODS

Experimental birds

Healthy White Leghorn laying and molting hens of approximately 500 days old were kept in individual cages under a daily light regimen of 14L:10D. The laying groups regularly laying 4 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 d of treatment. They were used after 20 d of cessation of egg-laying as the molting group. A proportion of the molting hens were intramuscularly injected daily with 1 mg of β-estradiol benzoate (Sigma-Aldrich Co., St. Louis, MO, USA; EB group) or 100 μL of sesame oil (control group) for 7 days. The birds were handled in accordance with the regulations of Hiroshima University for animal experiments.

Experiment 1. Analysis of mucin expression

The isthmus, uterus, and vagina of the laying (6 h after oviposition), molting, estradiol benzoate (EB), and control groups were collected after euthanization under anesthesia with Somnopentyl (Kyoritsu Seiyaku Inc., Tokyo, Japan) (n = 5 in each group). The average weights of oviducts were 95.8 ± 1.6 g, 7.9 ± 0.8 g, and 32.5 ± 0.9 g in laying, molting and EB-group, respectively.

Quantitative reverse-transcription PCR analysis for expression of mucin

Quantitative reverse-transcription PCR analysis was performed as described previously (Nii et al., 2011). Briefly, total RNA was extracted from the mucosal tissues of the isthmus, uterus and vagina using Sepasol RNA I Super (Nacalai Tesque Inc., Kyoto, Japan). The extracted total RNA samples were dissolved in TE buffer (10 mM Tris, pH 8.0, with 1 mM EDTA). They were treated with 1 U of RQ1 RNase-free DNase (Promega Co., Madison, WI, USA) on a PTC-100 programmable thermal controller (MJ Research Inc., Waltham, MA, USA), programmed at 37°C for 45 min and 65°C for 10

min. The concentration of RNA in each sample was measured using Gene Quant Pro (Amersham Pharmacia Biotech, Cambridge, UK).

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 the total RNA, 1 × RT buffer, 1 mM dNTP mixture, 20 U of RNase inhibitor, 0.5 μg oligo(dT)20 primer, and 50 U ReverTra Ace. Reverse transcription was performed at 42°C for 30 min, followed by heat inactivation for 5 min at 99°C using the PTC-100 Programmable Thermal Controller (MJ Research Inc.).

PCR was performed using Takara Taq (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. Primers used in this study are shown in Table 1. The PCR mixture (25 μL) contained 0.5 μL cDNA, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1.25 U Takara Taq, and 0.5 μM each primer. Mucin was amplified in a PTC-100 Programmable Thermal Controller (MJ Research Inc.) under the following conditions: 94°C for 30 sec, then 34 cycles at 95°C for 30 sec to denature, 58°C for 60 sec to anneal, 72°C for 60 sec for extension. The PCR products were separated by electrophoresis on a 2% (w/v) agarose gel containing 0.04% (w/v) ethidium bromide.

Real-time PCR was performed using the Roche Light Cycler system (Roche Applied Science, Indianapolis, IN). The reaction mixture (20 μL) contained 3 μL cDNA, 1 × SYBR Premix EX Taq (Takara, Tokyo, Japan) and 0.5 μM of each primer. The mixture was placed in 20 μL capillaries (Roche Diagnostics GmbH, Mannheim, Germany). The cycle parameters for the PCR reaction were 95°C for 5 sec and 62°C for 20 sec. Data analysis was performed as described by Livak and Schmittgen et al. (2001). Briefly, the Δ threshold cycle (CT) was calculated for each sample by subtracting the CT value of ribosomal protein S17 (RPS17) (internal control) from the CT of the respective target gene. For relative quantification, the ΔCT value of RPS17 was then subtracted from the ΔCT of each experimental sample to generate the ΔΔCT. The ΔΔCT value was therefore fit to the formula $2^{-\Delta\Delta CT}$ to calculate the approximate fold difference. The results were expressed as fold change obtained from the ratio of the experimental samples and a standard sample.

Experiment 2. Mucin immunohistochemistry

Tissue samples of the isthmus, uterus and vagina were fixed in 10% (v/v) formalin-PBS (n = 5; same birds used in Experiment 1), dehydrated and embedded in paraffin. Sections (4 μm thickness) were air-dried on slides. The deparaffinized sections were autoclaved in 10 mM sodium citrate, pH 6.0, for 1 min. After washing in PBS (3 times for 5 min each), the sections were incubated with 5% (v/v) normal goat serum for 30 min, followed by overnight incubation with the mouse anti-mucin5AC monoclonal antibody (Santa Cruz Biotech., Inc., Santa Cruz, CA, USA) diluted at a concentration of 4 μg/ml in PBS. After washing in PBS (3 times for 5 min each), the tissue sections were incubated with biotinylated anti-mouse IgG (Vector Lab. Inc., Burlingame, CA, USA) for 1 h. After washing in PBS (3 times for 5 min each), sections were incubated with avidin-biotin-peroxidase complex (Vector Lab. Inc.) for 1 h. After washing in PBS (3 times for 5 min each), the immunoreaction products were visualized using 0.02% (w/v) 3', 3'-diaminobenzidine-tetrahydrochloride and 0.005% (v/v) H_2O_2 in 0.05 M Tris-HCl, pH 7.6 (DAB-H_2O_2 mixture). Sections were counterstained with hematoxylin, dehydrated with graded series of alcohols and covered.

Alcian blue (AB) staining

Histochemical localization of mucin polysaccharide was performed by AB staining. Sections were deparaffinized and immersed into 3% (v/v) acetic acid for 1 min, followed by staining with 3% (v/v) AB dissolved in acetic acid for 1 h. After being washed in water, the sections were dehydrated and mounted.

Lectin histochemistry

To examine the localization of sugar residues in the mucosal barrier, the paraffin sections were stained by WGA or Jacalin lectins as described previously (Utsumi and Yoshimura, 2011) with minor modifications. Briefly, the sections were deparaffinized and incubated with 5% (v/v) normal goat serum for 30 min to block nonspecific staining. The tissue sections were incubated overnight with biotinylated WGA (J-Oil Mills, Inc., Tokyo, Japan) or biotinylated Jacalin (Vector Lab. Inc.) diluted at 20 μg/ml in PBS. After washing in PBS (3 times for 5 min each), sections were incubated with avidin-biotin-peroxidase complex (Vector Lab. Inc.) for 1 h. After

washing in PBS (3 times for 5 min each), lectin binding was visualized using a DAB-H_2O_2 mixture. Slides were counterstained with hematoxylin, dehydrated with graded series of alcohols and covered.

Statistical analysis

Fold changes in the mucin expressions were expressed as the mean ± SEM, and the significance of their differences between the laying and molting groups was examined by *t*-test. The significance of differences among laying, molting, and EB groups was examined by non-parametric analysis followed by Steel-Dwass test. Differences were considered significant at $P < 0.05$.

RESULTS

Histological observation showed that the surface of the mucosal epithelium in each oviductal segment was lined by ciliated pseudostratified epithelium and tubular glands were developed in the lamina propria of the isthmus and uterus of laying groups. In molting groups, the height of the mucosal epithelium was decreased in each segment and the tubular glands in the isthmus and uterus were regressed. These mucosal tissues were recovered in EB groups (Figure 4).

Experiment 1. Analysis of mucin expression

Figure 5 shows differences in the mucin mRNA expression in the vagina, uterus and isthmus between laying and molting groups. The expression was identified in all segments in laying groups; however, it was reduced markedly in molting group with significant differences between laying and molting groups within each segment.

Mucin gene expression in the mucosal tissue of laying, molting, and EB groups in the vagina and uterus is shown in Figure 6. In both the vagina and uterus, the relative expression level of mucin in the mucosal tissues was significantly lower in

molting group compared with laying group. The expression level was greater in the vagina and uterus of EB group than that in the molting group.

Experiment 2. Mucin immunohistochemistry

Figure 7 shows the localization of immunoreactive mucin5AC in the vagina, uterus and isthmus. In laying group, the immunoreaction products were identified on the surface of the mucosal epithelium of each segment (Figure 7a-c). Epithelial cells in each segment contained dense immunoreaction products in their cytoplasm (Figure 7a-c). Tubular gland cells in the uterus also contained immunoreaction products (Figure 7b). In contrast, in molting group, immunoreaction products on the surface of mucosal epithelium were negligible in the vagina, uterus and isthmus (Figure 7d-f). Immunoreaction products were observed in the cytoplasm of mucosal epithelial cells of the vagina (Figure 7d), whereas they were negligible in those of the uterus and isthmus (Figure 7e and f).

Figure 8 shows the localization of immunoreactive mucin5AC in the vagina, uterus and isthmus of control and EB groups. Immunoreaction products on the surface of mucosal epithelium were negligible in the vagina, uterus and isthmus in the control groups (Figure 8a, b, and c). In contrast, in EB group, immunoreaction products were identified on the surface of the mucosal epithelium of each segment (Figure 8d, e, and f). Epithelial cells in each segment contained dense immunoreaction products in their cytoplasm (Figure 8d, e, and f). Tubular gland cells in the uterus also contained immunoreaction products (Figure 8e).

Figure 9 shows the localization of Alcian blue (AB) positive substances in the vagina, uterus and isthmus of laying and molting groups. In laying group, the AB-positive substances were localized in the cells at the bottom of the secondary folds in the vagina, whereas it was negligible in the uterus and isthmus (Figure 9a, b, and c). In molting group, AB positive substances were negligible in the vagina, uterus and isthmus (Figure 9d, e, and f).

Figure 10 shows the localization of Alcian blue (AB) positive substances in the vagina, uterus and isthmus of control and EB groups. The AB positive substances were negligible in the vagina, uterus and isthmus in the control group (Figure 10a, b, and c).

In contrast, in EB group, AB positives substances were localized in in the cells at the bottom of the secondary folds in vagina, whereas it was negligible in the uterus and isthmus (Figure 10d, e, and f).

The results of WGA- and Jacalin-lectin histochemistry are shown in Figure 11. In laying group, WGA-positive and Jacalin-positive substances were localized in association with cilia on the surface of mucosal epithelium of the vagina, uterus and isthmus (Figure 11a-f). These positive substances were not observed at the pits of the secondary mucosal folds (Figure 11a-f). Substances positive for each WGA and Jacalin were also identified in molting hens (Figure 11d-f, j-l) as observed in laying group (Figure 11a-c, g-i).

DISCUSSION

In this Chapter it was examined whether the expression of mucin was affected by the egg-laying phase and estrogen in the lower oviductal segments (vagina, uterus, and isthmus) in hens. Significant findings were: (1) expression of mucin gene, immunoreactive mucin5AC, and AB-positive polysacharide in the lower oviductal segments was higher in laying hens than in molting group, (2) their expression was upregulated by estradiol, and (3) WGA- and Jacalin-positive substances were identified on the mucosal surface in both laying and molting groups.

It was observed that expression of mucin mRNA in the vagina, uterus and isthmus, and localized immunoreactive mucin5AC on the apical surface and in the cytoplasm of mucosal epithelium of these oviductal segments in laying hens. It is possible that the identified mucin5AC could be related to the expression of the mucin gene examined in this study. A fragment of chicken mucin cDNA prepared using the same primers as this study had 67% homology to human mucin5AC (Smirnov et al., 2004). The mucin mRNA and immunoreaction products of mucin5AC were markedly reduced or negligible in the mucosa in molting hens. These results suggest that mucin synthesis was reduced in the three lower oviductal segments during molting.

Mucin5AC, a gel-forming mucin, is the major constituent of mucus and confers its viscoelastic properties (Desseyn et al., 2000). It may prevent pathogen penetrance by inhibiting bacterial adhesion to the mucosal epithelium surface (Berry et al., 2002). Mucins are composed of a peptide core containing heavily glycosylated regions and

form a surface network above the epithelia (Carlstedt et al., 1983; Shambaugh et al., 1988). They have direct and indirect roles in defense from infection; namely, they have the ability to form a physical barrier and act as adhesion decoys (Linden et al., 2008a). The ability of the mucus layer to protect the epithelium from different pathogens is often attributed to the presence of charged groups in mucin molecules (Shambaugh et al., 1988). Not only do microbes bind to the sugar residue of mucin, but also, in some cases, mucins either have direct antimicrobial activity or carry other antimicrobial molecules (Linden et al., 2008b). Cell surface mucins may also initiate intracellular signaling in response to bacteria, suggesting that they have both a barrier and reporting function on the apical surface of mucosal epithelial cells (Linden et al., 2008a). The current study indicated that gene and protein expressions of mucin were significantly decreased in the lower segments of the oviduct in molting hens. These results suggest that the barrier function on the mucosal surface by mucin may be declined in the oviductal segments of molting hens.

Expression of mucin and the density of AB-positive substances were lower in the vagina, uterus, and isthmus of molting hens compared with laying hens. Treatment of molting birds with EB caused oviductal growth with gain of weight (Figure 1) and thickness of the surface epithelium. Mucin expression and density of AB-positive substances, namely mucopolysaccharides, was also increased in the surface epithelium in both the vagina and uterus by EB treatment. Thus, it was confirmed that estrogen causes the growth and differentiation of mucosal epithelial cells in the oviducts as has been described by many workers (Oka and Schimke, 1969; Berger and Sanders, 2000; Dougherty and Sanders, 2005). Estradiol-17β upregulated mucin-1, -4 and 5C expression in human endometrial cell line (Gollub et al., 1995; Paszkiewicz-Gadek et al., 2003). Thus, the upregulation of mucin expression in the female reproductive tract may occur commonly in birds and mammals. Smirnov et al. (2005, 2006) reported that mucin and mucin-2 expression was increased with development in chick small intestine. The increase of mucin expression in the mucosa in association with organ development seems a common feature among different organs. Probably, increase of goblet cell number and mucin synthesis activity is responsible for the elevated mucin expression in the mucosal tissues. The enhanced density of AB-positive substance in the surface epithelium suggests that goblet cells population and their activity for mucin synthesis were increased in the EB-treated molting hens. Thus, the current results suggest that mucin synthesis ability in the mucosal epithelium in

both vagina and uterus is upregulated in laying phase and estrogen plays a role in such development of mucin synthesis ability associated with oviductal growth and mucosal cell differentiation.

WGA- and Jacalin-lectin positive products were identified in association with the cilia on the surface of mucosal epithelium in both laying and molting groups. These results suggest that glycosylation by sugars recognized by these lectins occurs in substances on the surface of the mucosal epithelium, including mucin glycoprotein or the cell membrane. WGA binds to GlcNAc and sialic acid (Fallis et al., 2010; Tatsuzuki et al., 2009), whereas Jacalin binds to galactose and GalNAc (Kabir, 1998). The glycocalyx of the mucosal surface may act as an effective barrier against invasion by pathogenic microorganisms and injury from toxic substances (Buckley et al., 2000). Lectin histochemistry did not show differences in the localization of WGA- and Jacalin-positive substances in the vagina, uterus and isthmus between laying and molting groups. These sugar substances may be incorporated not only into mucin but also into other mucosal surface components and maintained even in the molting group.

In conclusion, mucins were expressed in mucosal tissue of the lower oviductal segment in laying group. Their expression declined in molting hens, but was upregulated by estrogen in association with epithelial differentiation and development in the vagina, uterus, and isthmus. Localization profile of the WGA- and Jacalin-positive sugar residue may not be changed in molting group. The reduction of mucin synthesis in the molting group may result in the decline of mucosal barrier functions, leading to greater susceptibility to pathogens than in the laying group.

ABSTRACT

Mucins play essential role as mucosal barrier to prevent invasion of pathogens in the oviductal tissue of hens. The aim of this study was to determine the effects of the egg-laying phase and estradiol on the mucin expression that forms a mucosal surface barrier in the lower oviductal segments in hens. White Leghorn laying and molting hens were used. Molting hens were given either sesame oil (control group) or estradiol benzoate (EB group) via i.m. injection (n = 5 per group). The lower segments of oviduct (vagina, uterus, and isthmus) of these birds were collected. Localization and gene expression of mucosal mucin were analyzed by quantitative RT-PCR and immunohistochemistry. Localization of mucin polysaccharide was performed by alcian blue (AB) staining. Sugar residues were localized by lectin (WGA or Jacalin) histochemistry. In the vagina, uterus and isthmus, mucin expression was formed, and immunoreactive mucin5AC and AB-positive mucopolysaccharide were localized in the mucosal epithelium. Their expression and density were lower in molting hens compared with laying hens, and up-regulated by EB. Substances positively stained by WGA and Jacalin were identified on the surface of the mucosal epithelium in the lower oviductal segments in laying and molting hens. These results suggest that mucin synthesis in the lower segments of the oviduct is reduced due to decline of circulating estrogen level, although the existence of WGA- and Jacalin-positive sugars may be kept even in the molting phase. The reduction of mucin synthesis may result in a decline of mucosal barrier function in the molting group.

Table 1. Primer sequences for *mucin* and *ribosomal protein S17 (RPS17).*

Target genes	Sequences 5' – 3'	Accession number References
Mucin	F: TCT TCC GCT ACC CTG GGC TCT GTAA	GI_45125071
	R: CTC ATG CAG TTC TAG CAA GAT ACT	Smirnov et al., 2004
RPS17	F: AAG CTG CAG GAG GAG GAG AGG	NM_204217
	R: GGT TGG ACA GGC TGC CGA AGT	Nii et al., 2011

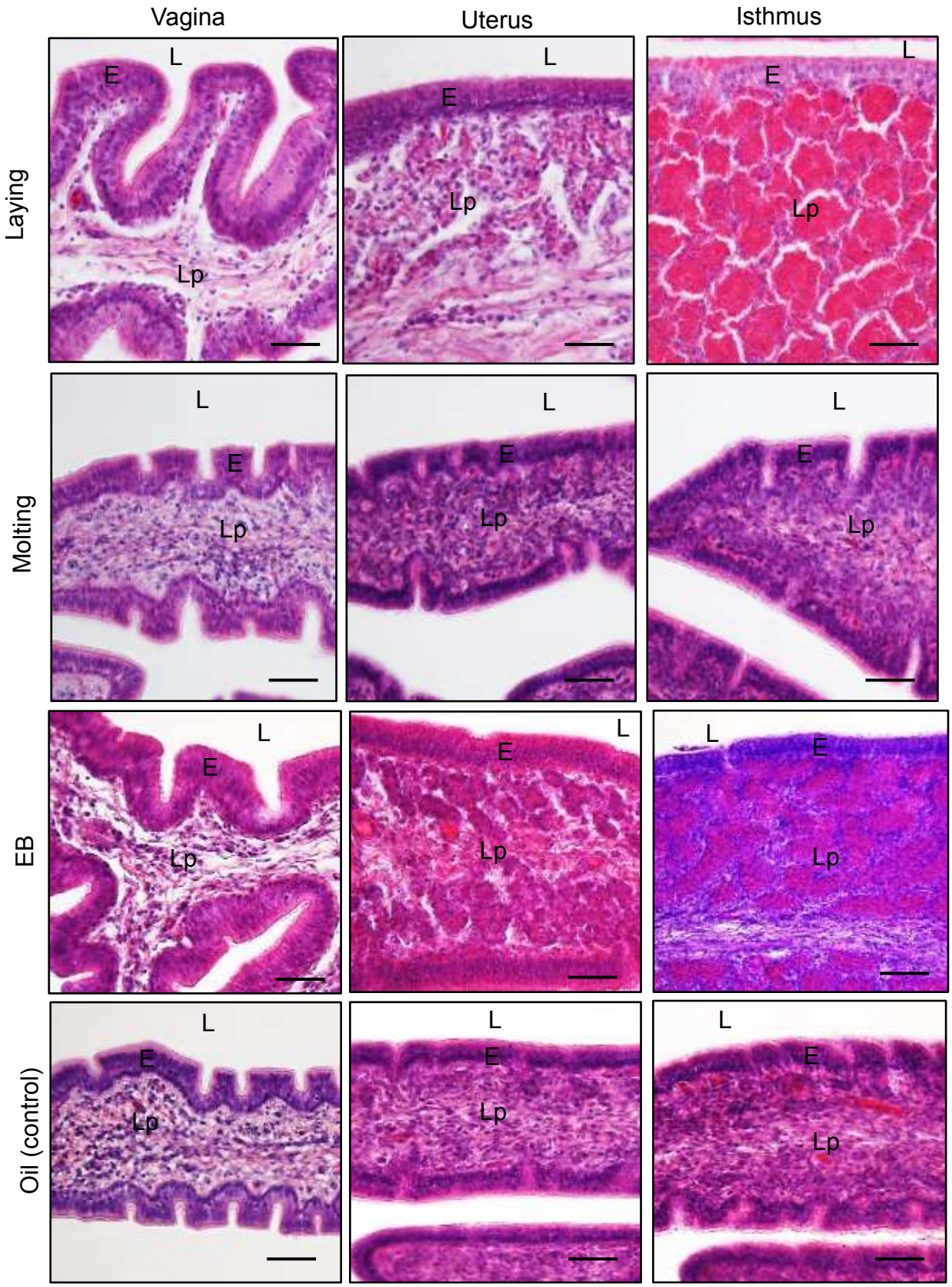

Figure 4. Histological observation with hematoxylin–eosin (HE) staining in the vagina, uterus and isthmus of laying, molting, EB, and control groups. EB = estradiol benzoate, E = mucosal epithelium, L = lumen of oviduct, Lp = lamina propria. Scale bars = 50 μm.

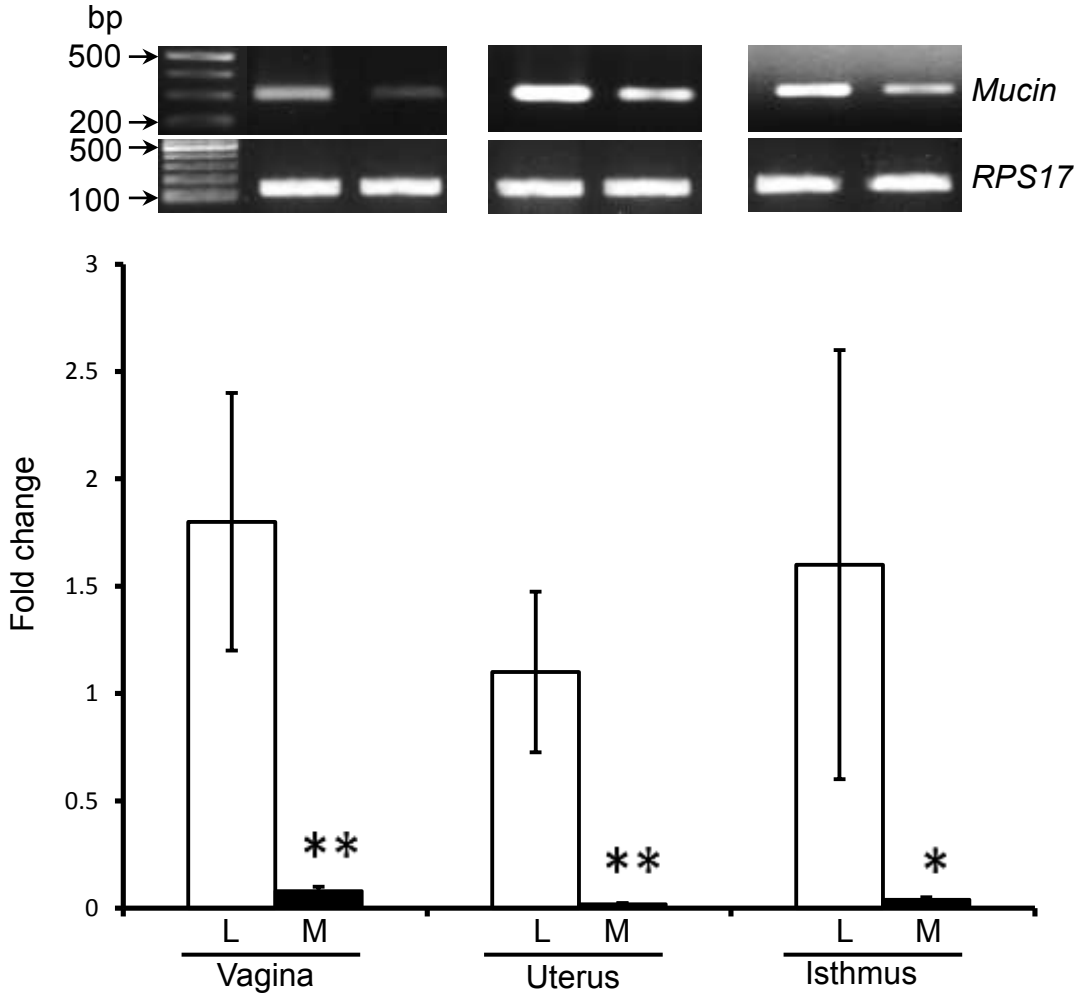

Figure 5. Differences in mucin mRNA expression in the vagina, uterus and isthmus between laying (L) and molting (M) groups. Values are the mean ± SEM of fold change (n=5). Asterisks indicate that values are significantly different between laying and molting groups (* $P<0.05$, ** $P<0.01$; *t*-test). L = laying group, M = molting group, RPS17 = ribosomal protein S17.

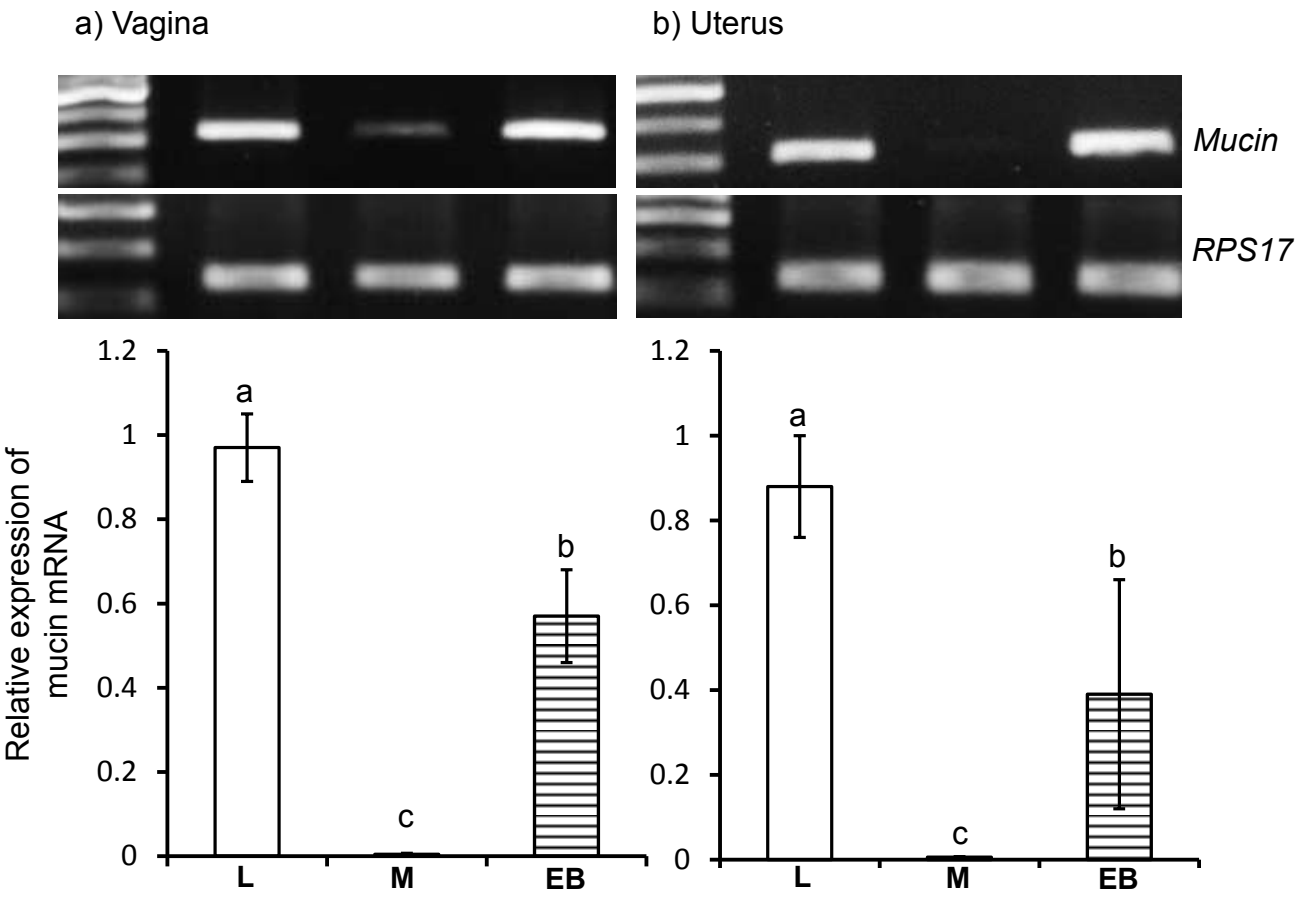

Figure 6. Mucin gene expression in the mucosal tissue of laying (L), molting (M), and EB groups in the vagina and uterus. In both the vagina and uterus, the relative expression level of mucin in the mucosal tissues is significantly decreased in molting group compared with laying group. The expression level is greater in the vagina and uterus of EB group than that in the molting group. Values are the mean ± SEM of relative expression (n = 5). EB = estradiol benzoate, RPS17 = ribosomal protein S17. ^{a,b,c} Values are significantly different among laying, molting, and EB groups ($P<0.01$; Steel-Dwass test).

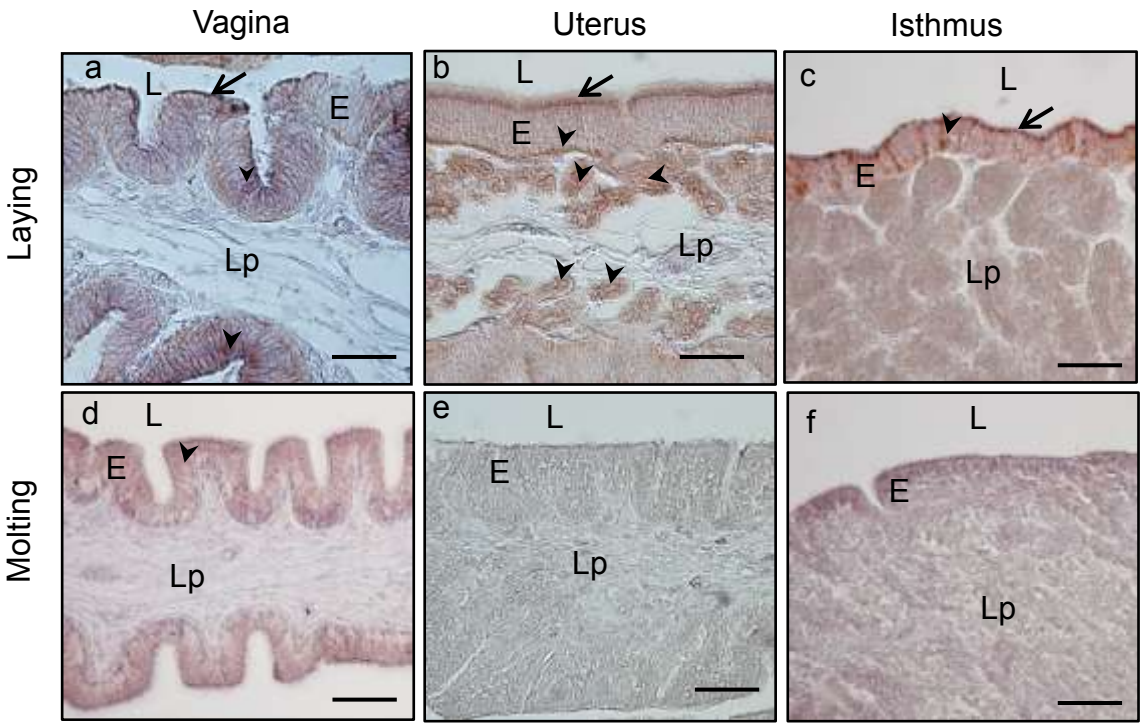

Figure 7. Localization of immunoreactive mucin5AC in the vagina, uterus and isthmus of laying and molting groups, (a-c = laying group, d-f = molting group; a and d = vagina, b and e = uterus, c and f = isthmus). In laying group, immunoreaction products are identified on the surface of mucosal epithelium of each segment (arrows). Epithelial cells of each segment and tubular gland cells in the uterus contain immunoreaction products in their cytoplasm (arrowheads). In molting group, immunoreaction products on the surface of mucosal epithelium are negligible in each segment. E = mucosal epithelium, L = lumen of oviduct, Lp = lamina propria. Scale bars = 50 μm.

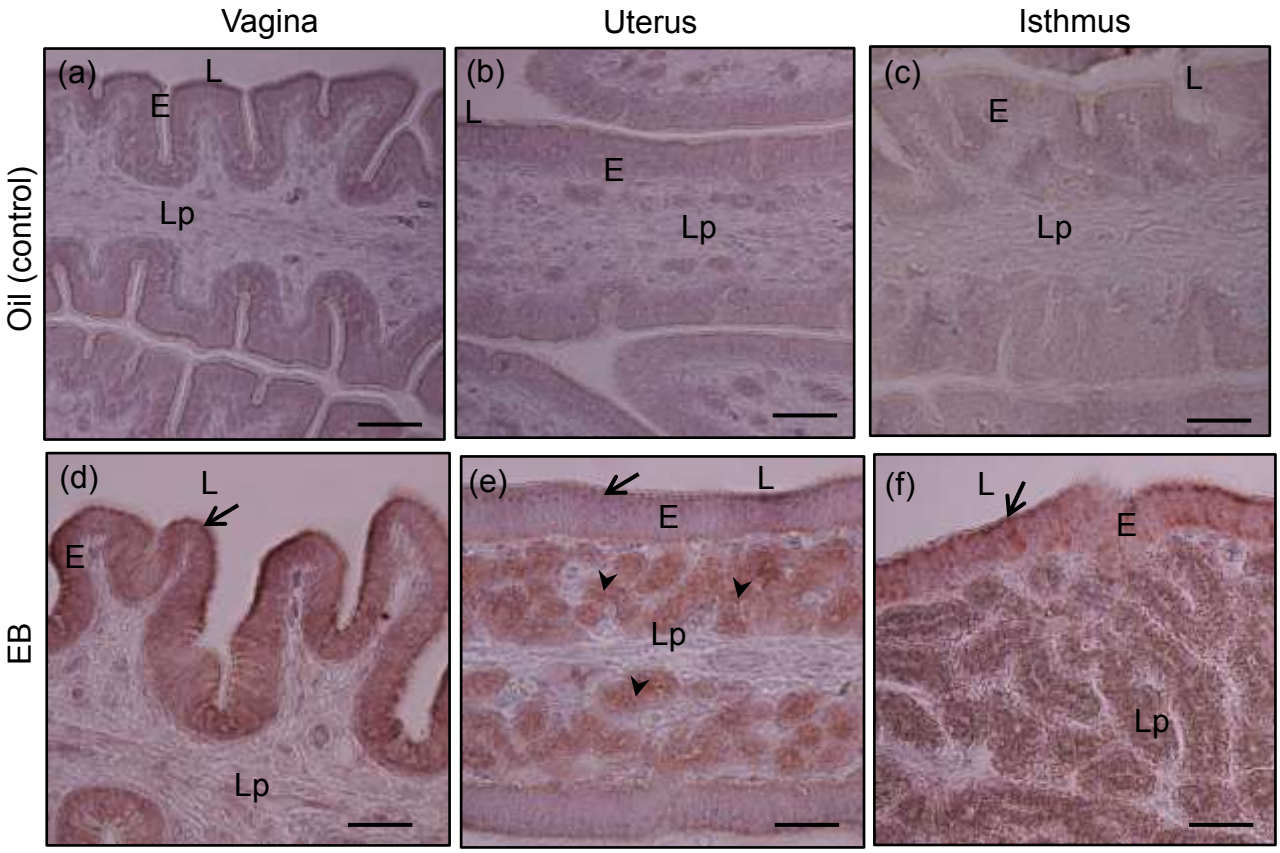

Figure 8. Localization of immunoreactive mucin5AC in the vagina, uterus and isthmus of oil (control) and EB groups, (a-c = control group, d-f = EB group; a and d = vagina, b and e = uterus, c and f = isthmus). In control group, immunoreaction products on the surface of mucosal epithelium are negligible in each segment. In EB group, immunoreaction products are identified on the surface of mucosal epithelium of each segment (arrows). Epithelial cells of each segment and tubular gland cells in the uterus contain immunoreaction products in their cytoplasm (arrowheads). EB = estradiol benzoate, E = mucosal epithelium, L = lumen of oviduct, Lp = lamina propria. Scale bars = 50 μm.

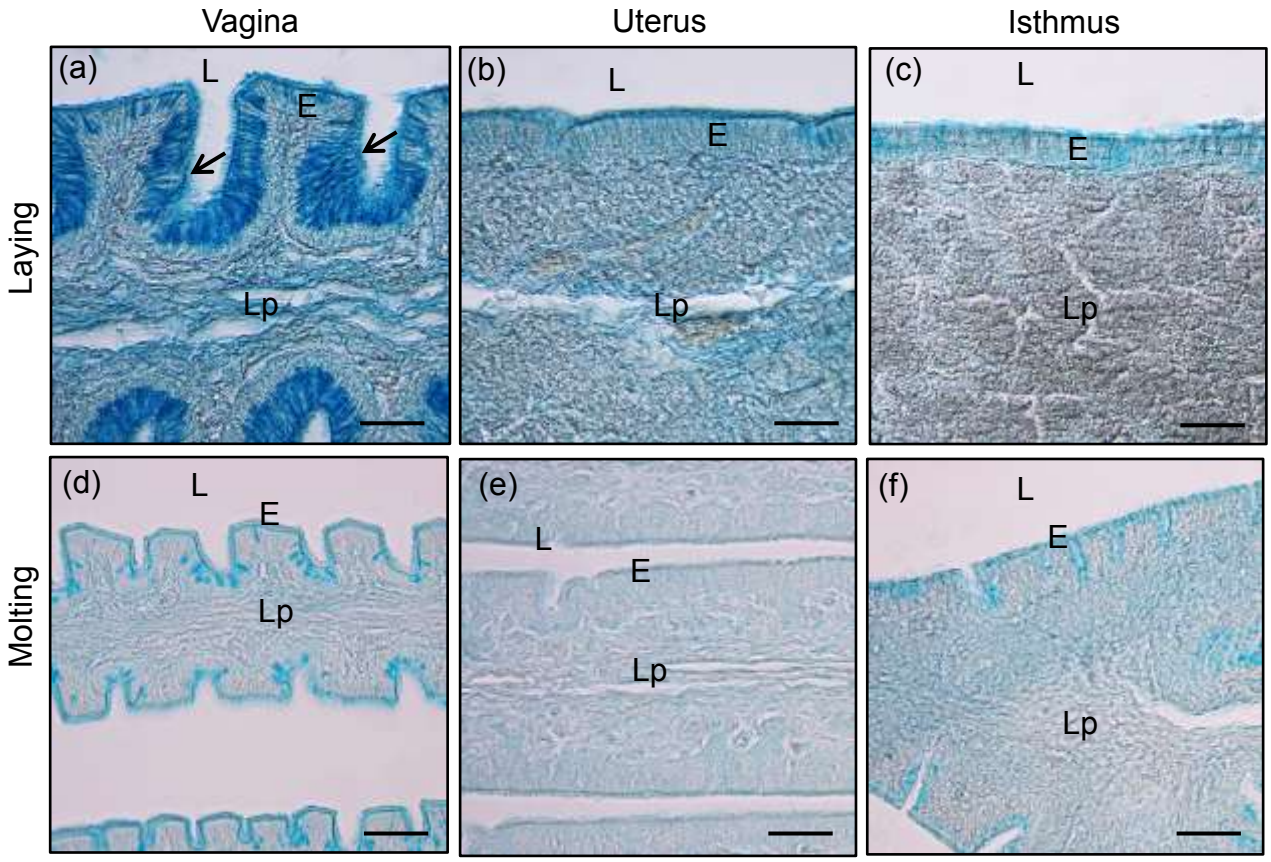

Figure 9. Localization of Alcian blue (AB) positive substances in the vagina, uterus and isthmus of laying and molting groups. The mucosal tissues are well developed with formation of secondary folds and high epithelial cells lining the surface. The AB-positive substances are localized in the cells at the bottom of the secondary folds in vagina of laying group (a), its density is reduced in the molting group (d). In both laying and molting groups, positive substance on mucosal epithelium of uterus and isthmus are negligible in each segment. E = mucosal epithelium, L = lumen of oviduct, Lp = lamina propria. Scale bars = 50 μm.

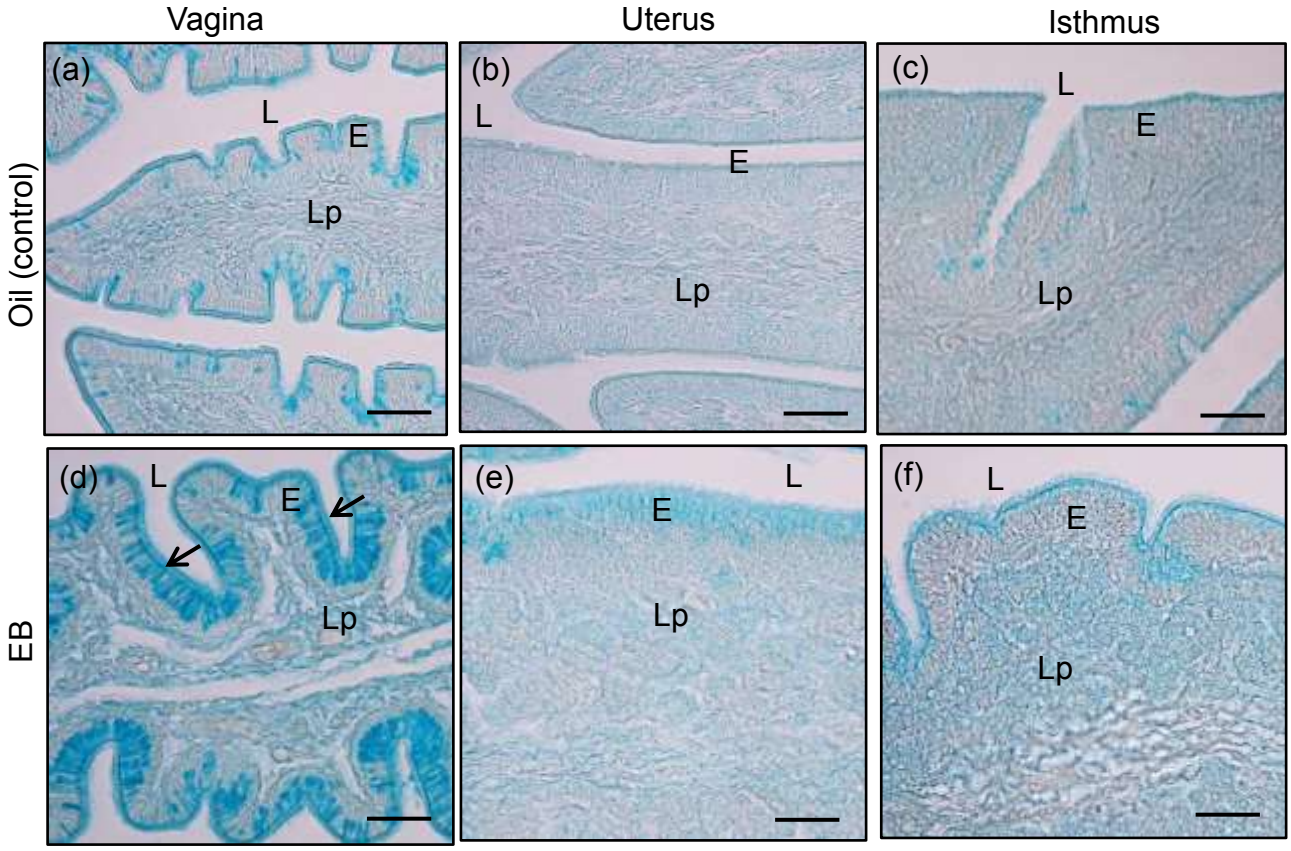

Figure 10. Localization of Alcian blue (AB) positive substances in the vagina, uterus and isthmus of oil (control) and EB groups. The AB-positive substances are negligible in each segment of the control groups (a-c). In the EB group, the density is increased in the vagina (d), whereas it does not affect in uterus and isthmus (e and f). EB = estradiol benzoate, E = mucosal epithelium, L = lumen of oviduct, Lp = lamina propria. Scale bars = 50 μm.

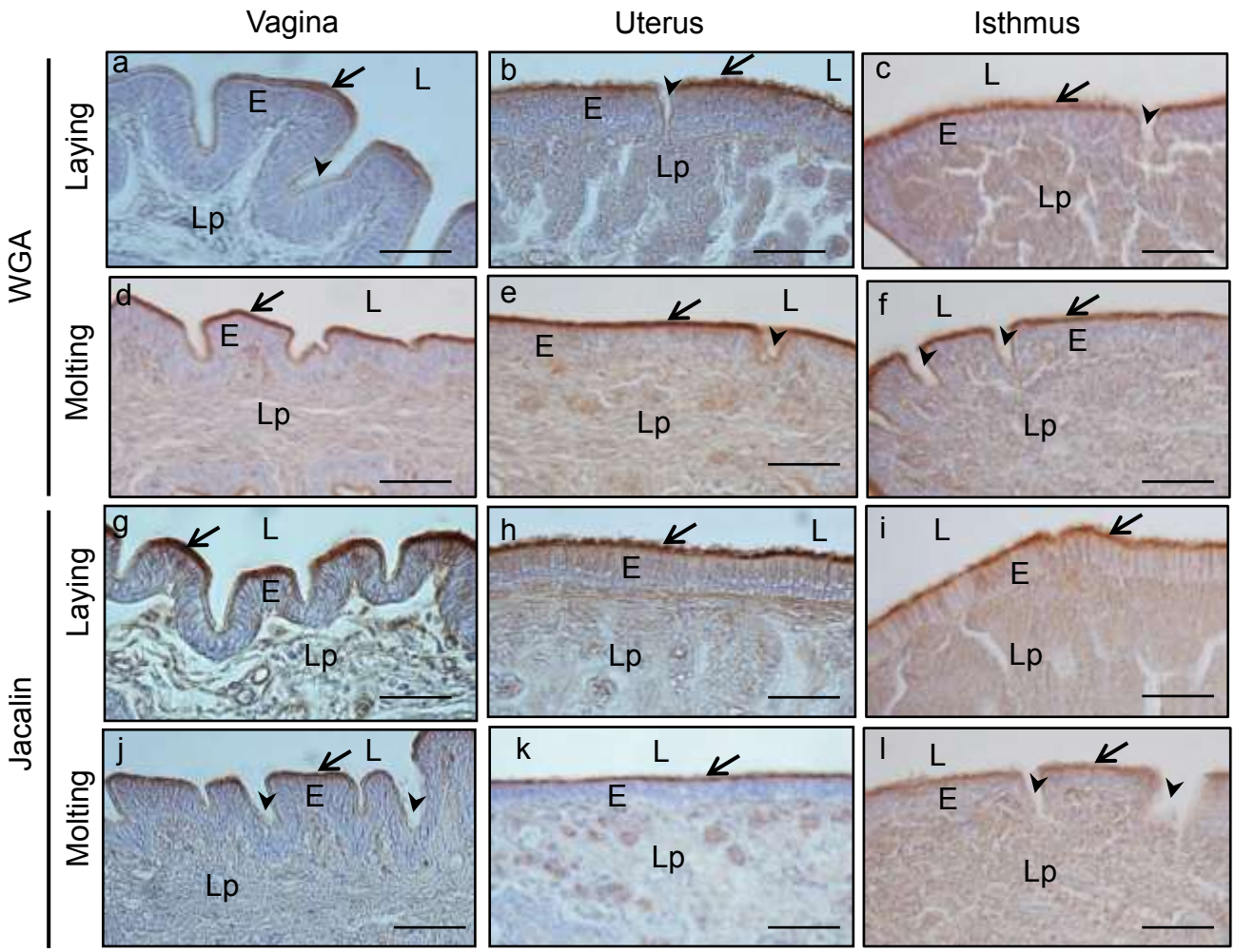

Figure 11. Lectin histochemistry using WGA and Jacalin in the vagina, uterus and isthmus of laying (a-c, g-i) and molting groups (d-f, j-l). a-f = WGA staining; g-l = Jacalin staining; a, d, g, j = vagina; b, e, h, k = uterus; c, f, i, l = isthmus. Both WGA-positive and Jacalin-positive substances are localized in association with cilia on the surface of mucosal epithelium of each segment of laying and molting groups (arrows). Positive substances are not observed in the pits of the secondary mucosal folds (arrowheads). E = mucosal epithelium, L = lumen of oviduct, Lp = lamina propria. Scale bars = 50 μm.

Chapter 3

INDUCTION OF MUCIN EXPRESSION BY LIPOPOLYSACCHARIDE IN THE LOWER OVIDUCTAL SEGMENTS IN HENS

INTRODUCTION

Mucosal barrier systems formed by mucus gel, epithelial cell junctional structures, and leukocyte activity, play essential roles to prevent infection by pathogenic agents in the mucosal tissue of hen oviducts. Mucins produced by goblet cells in the epithelium or submucosal glands are the major mucus macromolecules responsible for the viscous properties of the mucus gel (Gendler and Spicer, 1995; Linden et al., 2008b). They are the first barriers that cover the mucosal surface protecting the epithelial cells from chemical, enzymatic, microbial, and mechanical damage. They have direct and indirect roles in defense from infection; namely, the ability to form a physical barrier and to act as adhesion decoys. Cell surface mucins may also initiate intracellular signaling in response to bacteria, suggesting that they have both a barrier and reporting function on the apical surface of mucosal epithelial cells (Corfield et al., 2000; Linden et al., 2008b).

The results of Chapter 2 showed that mucosal barrier formed by mucin is affected by egg-laying phases, namely, laying and molting phase. Previous studies in chicks showed increased mucin mRNA expression in the intestine by starvation (Smirnov et al., 2004), probiotic feeding (Smirnov et al., 2005), and *in ovo* administration of carbohydrates (Smirnov et al., 2006). Immunoreactive mucins including mucin5AC, a secreted gel-forming mucin, in the distal colonic epithelium of mice were reported to be decreased by infection with *Citrobacter rodentium* (Linden et al., 2008a). Mucin5AC expression was up-regulated by bacterial lipopolysaccharide (LPS) in human goblet cells (Smirnova et al., 2003) and in human nasal epithelium (Wang et al., 2013). Thus, mucin synthesis may be affected by physiological and environmental factors in mucosal tissues.

The hen oviduct could be infected by *Salmonella enteritidis* (Keller et al., 1995; Takata et al., 2003; De Buck et al., 2004a). Previous reports suggested that the oviduct was more susceptible in molting hens than in laying hens because contamination of eggs by *Salmonella* was more frequent after resumption of laying in postmolting hens (Golden et al., 2008). If the expression of mucins in the oviductal epithelium declines, mucosal barrier functions may be weakened, leading to increased susceptibility to pathogenic agents. In Chapter 2, it was suggested that expression of mucins was higher in laying hens than in molting hens, and upregulated by estrogen. However, it is unknown whether mucin expression is induced by LPS and the responses to LPS for the mucin expression are changed by egg-laying phase (laying and molting) and estrogen.

The aim of this study was to determine the effect of LPS on the mucin expression in the lower oviductal segments (vagina and uterus) of hens. Specific questions were asked as to whether: (1) LPS stimulation induced mucin expression in laying and molting hens, and (2) estrogen affected sensitivity of mucosal tissues to LPS for mucin induction in those oviductal segments.

MATERIALS AND METHODS

Experimental birds

White Leghorn hens, approximately 500-d old and laying 4 or more eggs in a clutch, were used. They were kept under a same condition as Chapter 2. Molting was induced by restricted feeding (25 g/d) as described in Chapter 2. The hens were euthanized under anesthesia with Somnopentyl (Kyoritsu Seiyaku Inc.) for sample collection. The birds were handled in accordance with the regulations of Hiroshima University for animal experiments.

Effects of LPS on the mucin expression in the lower oviductal segments of laying, molting and EB groups were examined *in vitro* (n = 5 each). In the EB group, molting birds were intramuscularly injected for 7 d with 1 mg β-estradiol-benzoate (Sigma Co) as described in Chapter 2 that showed a significant development of the oviduct. The average weights of oviducts were 95.8 ± 1.6 g, 7.9 ± 0.8 g, and 32.5 ± 0.9

g in laying, molting and EB-group, respectively. The mucosal tissues of the vagina and uterus were collected, and cut into small pieces (approximately 1 x 1 x 5 mm) and placed in sterile tubes for culture (3 tissue pieces per tube) (Greiner Bio-one, Ltd., Tokyo, Japan) containing 4 ml culture medium with or without LPS. The culture medium was TCM-199 medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10% bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), 100 U/ml penicillin and 100 µg/ml streptomycin (Cosmo Bio, Tokyo, Japan). For dose dependency analysis, the tissues were incubated in the medium with LPS at concentrations of 10, 100, 1000 ng/ml for 3 h. Stock solution of LPS was prepared by dissolving LPS (lipopolysaccharide from *Salmonella minnesota*; Invivogen, San Diego, CA, USA) in PBS at a concentration of 5 mg/ml, and added to the culture medium according to the designed working concentration. Corresponding volume of PBS was added to the medium for control group. For time course analysis, tissues were incubated with 100 ng/ml LPS for 0, 1.5, 3 h. Incubation was performed in a CO_2 incubator at 37°C under a humidified atmosphere with 5% CO_2 and 95% air.

Quantitative RT-PCR analysis for expression of mucin

Expression of mucin in the cultured tissues was analyzed by quantitative RT-PCR as described in Chapter 2. Briefly, total RNA was extracted from cultured tissues. Then, the RNA samples were reverse-transcribed to obtain cDNAs. PCR and real time PCR for analysis of mucin expression were performed using same primers and protocols as described in Chapter 2. Real-time PCR data were analyzed by the $2^{-\Delta\Delta CT}$ method to calculate the relative level of mRNA in each sample using *RPS17* for the housekeeping gene (Livak and Schmittgen, 2001). Samples of the vagina and uterus of laying, molting and EB-group hens before incubation were used for analysis of the dose and time dependency effect of LPS. The vagina and uterus samples of a laying hen incubated with the solution including 100 ng/ml LPS for 3 h were also used as the standard for comparison of LPS effect among laying, molting, and EB-group hens. The results were expressed as relative expression obtained from the ratio between the experimental samples and the standard sample.

Alcian blue (AB) staining

The cultured tissue samples of the uterus and vagina were fixed with 10% (v/v) formalin in PBS, and processed for paraffin sections (4 μm in thickness). Mucin polysaccharide was localized by AB staining as described in Chapter 2.

Statistical analysis

The significance of the differences among different LPS dose groups as well as among different incubation time groups was examined by non-parametric analysis followed by Steel-Dwass test. The significance of the differences between LPS-treated and control groups was examined by Student's *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

Figure 12 shows the effects of LPS on the mucin expression in the cultured mucosal tissue of the vagina and uterus of laying groups. The expression level was increased 4.5 to 7 folds in the vagina (Figure 12a) and 5 to 23 folds in the uterus (Figure 12c) by stimulation with 10 to 1,000 ng/mL LPS for 3 h. The expression level was significantly greater in the groups stimulated by LPS at 10, 100 or 1,000 ng/ml than in the control group in both the vagina and uterus (Figure 12a, c). The increase of the expression level by 100 ng/ml LPS was observed after 1.5 and 3 h incubation in both the vagina and uterus (Figure 12b, d).

The effects of LPS on mucin expression in the cultured mucosal tissue of molting groups are shown in Figure 13. The expression was increased in a LPS dose dependent manner at 10 to 1,000 ng/ml in both the vagina and uterus (Figure 13a, c). The expression was higher in the vaginal tissues treated with LPS at 10, 100 and 1,000 ng/ml (Figure 13a), and in the uterus tissues treated with LPS at 100 and 1,000 ng/ml compared with the control group (Figure 13c). The expression level was also increased by LPS in a time dependent manner at 1.5 h and 3 h.

Figure 14 shows the effects of LPS on mucin expression in the mucosal tissues of EB groups. Unlike laying and molting groups, LPS did not affect the expression at

any doses and time course, and there was no difference in the expression between control and LPS treated groups in both the vagina and uterus (Figure 14a, c).

The differences in mucin expression in the cultured mucosal tissues stimulated by LPS (100 ng/mL, 3 h) among laying, molting and EB groups are shown in Figure 15. The expression was significantly lower in EB groups than in laying and molting groups within the vagina (Figure 15a) or uterus (Figure 15b).

Localization of Alcian blue (AB) positive substances in the vagina of laying, molting, and EB groups treated with or without LPS are shown in Figure 16. The mucosal tissues were well developed with formation of secondary folds and high epithelial cells lining the surface. The AB-positive substances were localized in the cells at the bottom of the secondary folds in laying groups (Figure 16a). The density was reduced in the molting groups (Figure 16d), and recovered in the EB groups (Figure 16g). Treatment of the tissue with LPS did not affect the localization and density of the AB-positive substance in each group (Figure 16b, c, e, f, h, and i). The effects of LPS on the AB-positive substances were negligible also in the uterine mucosa (data not shown).

DISCUSSION

In this Chapter, it was examined whether LPS affects the mucin expression in the vagina and uterus of hens. Major finding was that LPS induced mucin expression in laying and molting hens but not in the estrogen-treated hens. The mucosal barrier of the vagina and uterus provides protection from infection by microorganisms ascending from the cloaca. Treatment with LPS stimulated mucin expression in the mucosal tissue of the vagina and uterus of both laying and molting hens. This result suggests that mucin protein synthesis in the vagina and uterus is activated in response to LPS of Gram-negative bacteria when they infect the mucosal surface. The induction of mucin transcription and overproduction in the epithelial cells of the airways by LPS has also been reported in mammals (Song et al., 2009; Wang et al., 2009). However, the differences in the density of AB positive substances, namely mucopolysaccharide, were negligible between LPS stimulated and unstimulated tissues. Histochemical

analysis by AB could localize the mucopolysaccharide clearly, but may not be enough to show the differences in the density of positive substance. It remains unknown whether LPS affects the synthesis of mucopolysaccharides.

The LPS is a ligand of Toll-like receptor (TLR) 4, and upregulated the cytokine expression and recruited T cells in hen oviduct (Nii et al., 2011; 2013). Previous study identified expression of TLRs including TLR4 in hen oviduct (Ozoe et al., 2009). In the downstream of TLRs, signal transduction molecules involved in MyD88 dependent or independent pathway play roles to regulate the nuclear transcriptional factors such as nuclear factor kappa B (NFκB) and activated protein-1 (AP-1) (Ghisletti et al., 2005; Kogut et al., 2012). Wang et al. (2013) reported that LPS treatment activated NFκB and promoted its nuclear translocalization to induce mucin (*MUC5AC*) overproduction in human nasal epithelial cells. It is assumed that LPS was recognized by TLR4, which activated NFκB to induce mucin also in the mucosa of the vagina and uterus as will be examined in the later Chapter.

The oviducts were grown and the mucosal epithelium of the vagina and uterus was differentiated by EB. However, LPS did not induce mucin expression in the EB-group hens, and the mucin expression level after LPS treatment was lower in EB-group hens than in laying and molting hens. Ghisletti et al. (2005) reported that in mouse macrophages estradiol-17β blocked LPS-induced DNA binding and transcriptional activity of p65, a member of NFκB, by preventing its nuclear translocation. Since the LPS-induced mucin expression is reported to be mediated by NFκB (Wang et al., 2013), prevention of activated NFκB by estrogen may affect the induction of mucin by LPS. It is assumed that the suppression of LPS-induced mucin expression by EB observed in this study is due to such prevention of NFκB nuclear translocation by EB. Thus, estrogen may not be a major factor for the development and maintenance of intracellular signaling pathway for induction of mucin expression by LPS, namely response to LPS, in the vagina and uterus. The effects of progesterone on the mucin expression and regulation of intracellular signaling have not been examined. Role of progesterone in those cellular functions for mucin expression should be examined by future studies.

In conclusion, the current results suggest that bacterial LPS stimulated mucin expression in those epithelial cells of both laying and molting hens, which may enhance mucosal barrier function and play role in preventing infections.

ABSTRACT

Mucins play essential role as mucosal barrier to prevent invasion of pathogens in the oviductal tissue of hens. The aim of this study was to determine the effect of lipopolysaccharide (LPS) on the mucin expression in the lower oviductal segments (vagina and uterus) of hens. The mucosal tissues of the vagina and uterus were collected from White Leghorn laying and molting hens, and molting hens with or without intramuscular injection with 1 mg estradiol-benzoate (EB) daily for 7 d. These tissues were cultured in TCM-199 culture medium with or without LPS (0, 10, 100 or 1000 ng/ml) for 1.5 or 3 h. Mucin expression in those tissues cultured with or without LPS was analyzed by quantitative RT-PCR. Cultured tissues were also processed for paraffin sections and stained with Alcian blue (AB). Mucin expression in the cultured vagina and uterus tissues of laying and molting hens was up-regulated by LPS in a dose- and time-dependent manner. However, there was no significant response to LPS for induction of mucin in the tissues of EB-group hens. The mucin expression level in the vagina and uterus tissues stimulated by LPS was lower in the EB-group hens than in laying and molting hens, and also that in the uterus was lower in the molting hens than in laying hens. These results suggest that mucin expression responsible for mucosal barrier is stimulated by LPS in the vagina and uterus of both laying and molting hens. Estrogen may suppress the response to LPS for mucin induction. The LPS-induced mucin expression may enhance mucosal barrier function and play a role in preventing infections by bacteria in the vagina and uterus.

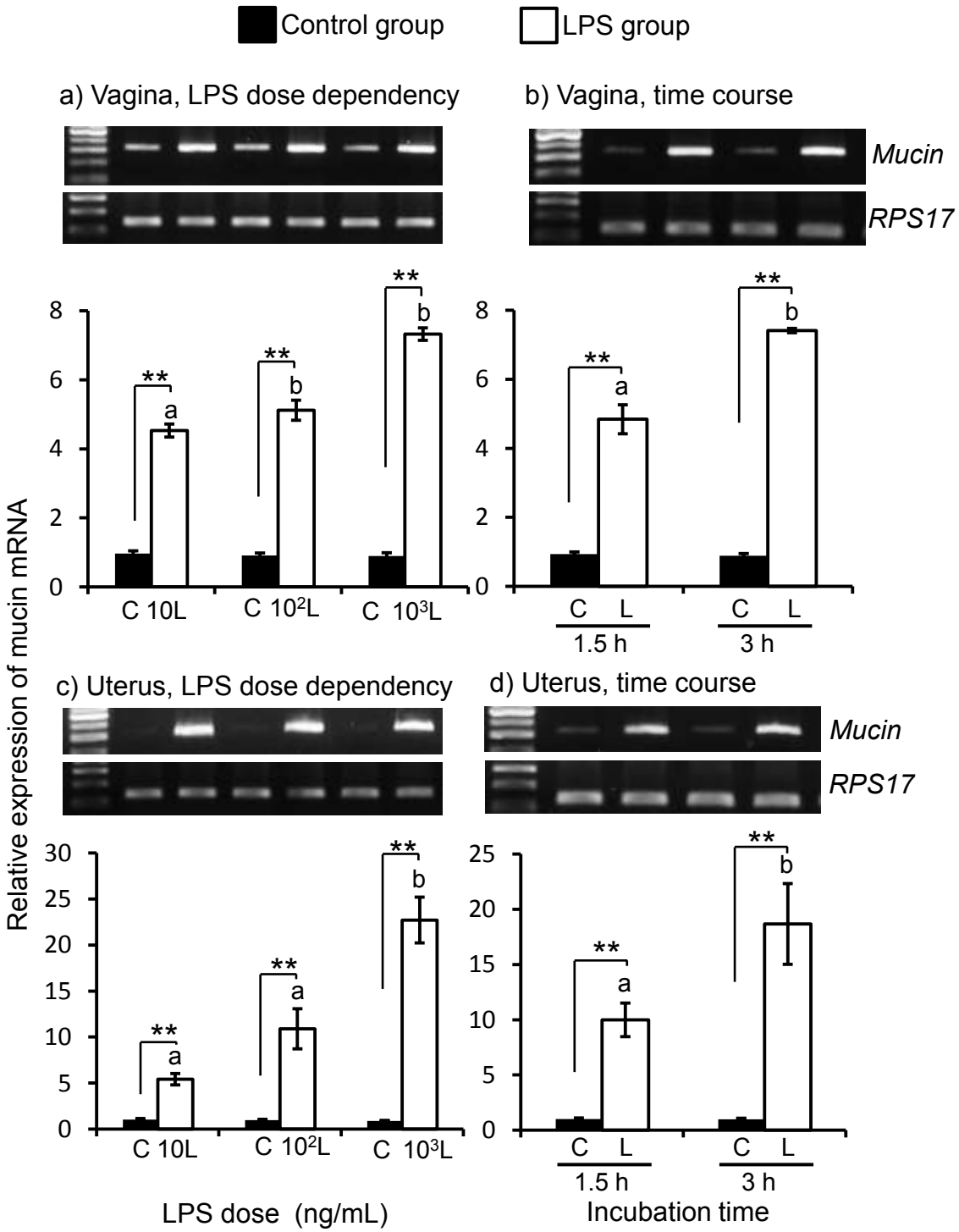

Figure 12. Effects of LPS on the mucin expression in the cultured mucosal tissue of the vagina and uterus of laying group. Values are the mean ± SEM of relative expression (n=5). ** Values are significantly different between LPS and control groups ($P<0.01$; *t*-test). [a,b,c] Values are significantly different among different LPS doses groups as well as among different incubation time groups ($P<0.05$; Steel-Dwass test). The tissues of LPS group and control group were incubated for 3 h with or without LPS. 10L, 10^2L and 10^3L = groups treated with 10, 100 and 1000 ng/mL LPS, respectively. LPS = lipopolysaccharide, RPS17 = ribosomal protein S17.

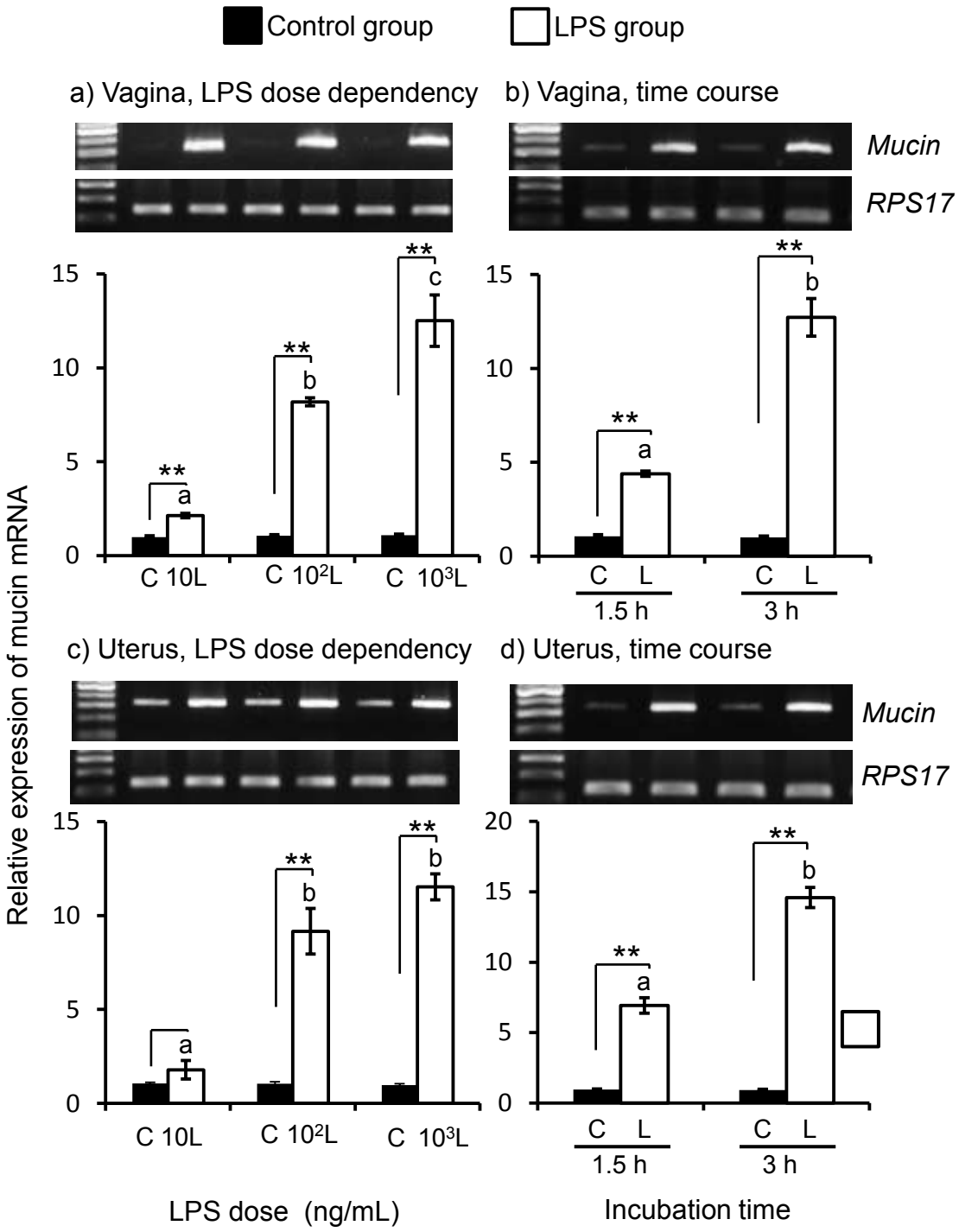

Figure 13. The effects of LPS on mucin expression in the cultured mucosal tissue of molting group. Values are the mean ± SEM of relative expression (n=5). ** Values are significantly different between LPS and control groups ($P<0.01$; *t*-test). [a,b,c] Values are significantly different among different LPS doses groups as well as among different incubation time groups ($P<0.05$; Steel-Dwass test). LPS = lipopolysaccharide, RPS17 = ribosomal protein S17. See Figure 12 for explanation of 10L, 10^2L and 10^3L.

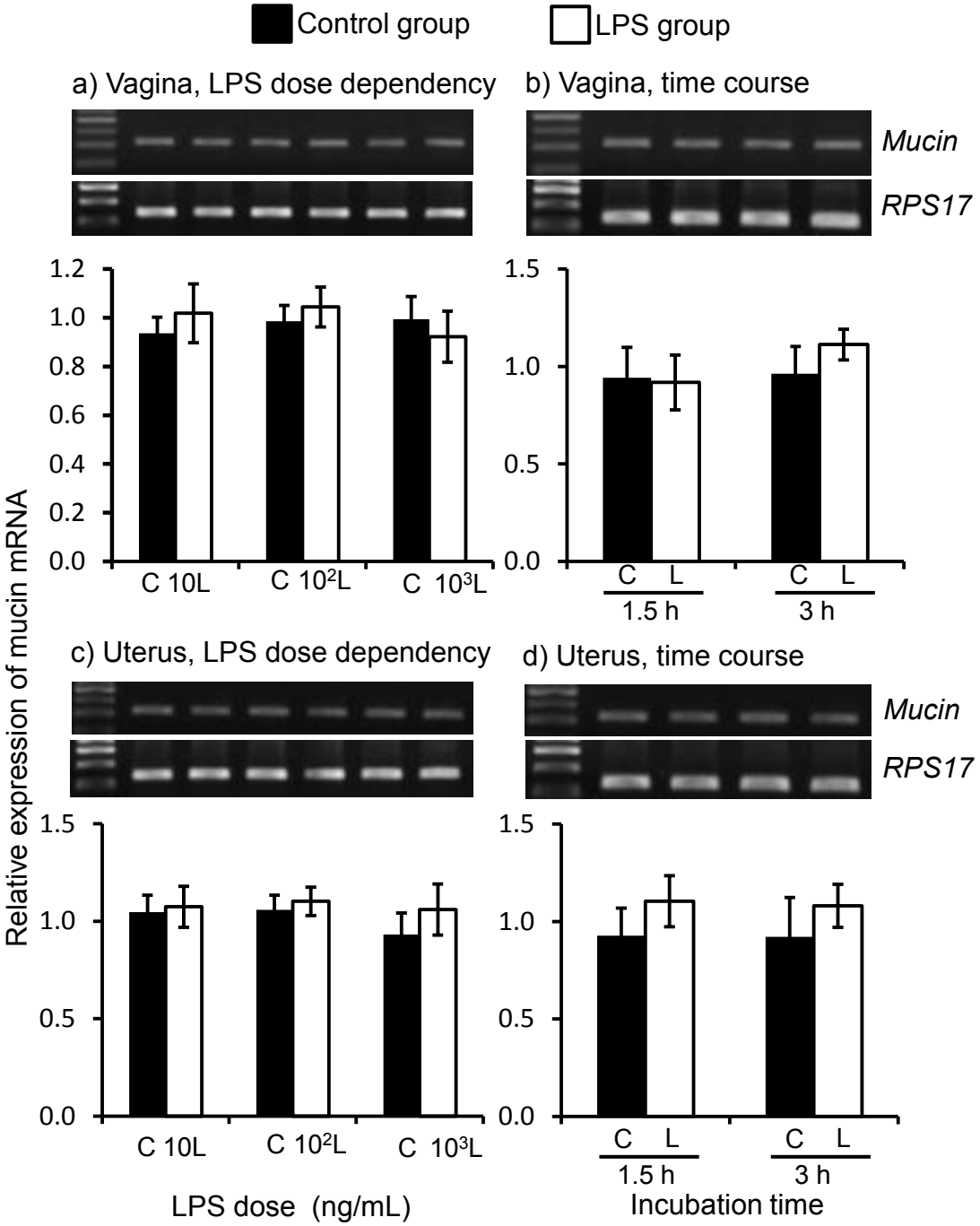

Figure 14. Effects of LPS on mucin expression in the mucosal tissues of EB group. Values are the mean ± SEM of relative expression (n=5). EB = estradiol benzoate, LPS = lipopolysaccharide, RPS17 = ribosomal protein S17. See Figure 12 for explanation of 10L, 10^2L and 10^3L.

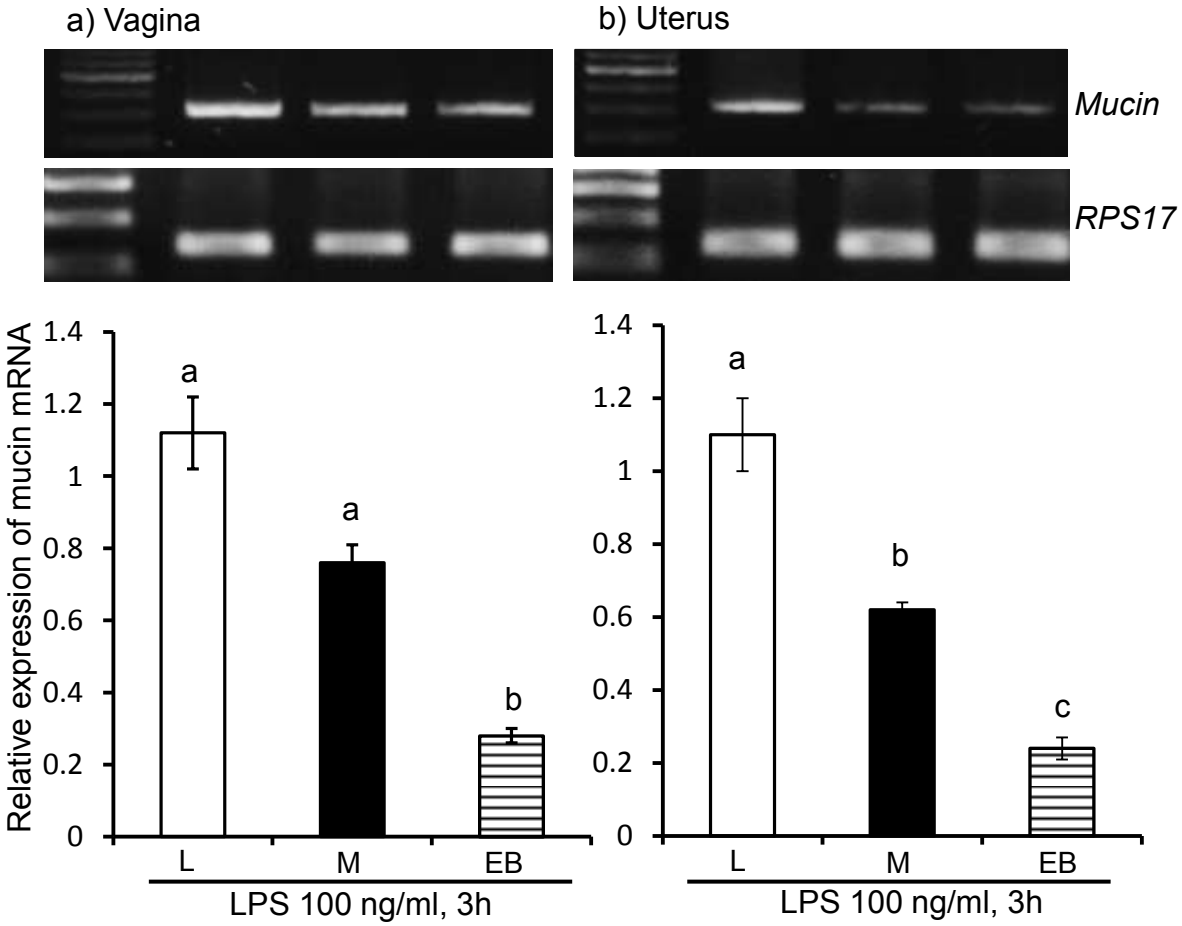

Figure 15. The differences in mucin expression in the cultured mucosal tissues stimulated by LPS (100 ng/mL, 3 h) among laying (L), molting (M), and EB groups. Values are the mean ± SEM of relative expression (n=5). [a,b,c] Values are significantly different among laying, molting, and EB-group hens ($P<0.01$; Steel-Dwass test). EB = estradiol benzoate, LPS = lipopolysaccharide, RPS17 = ribosomal protein S17.

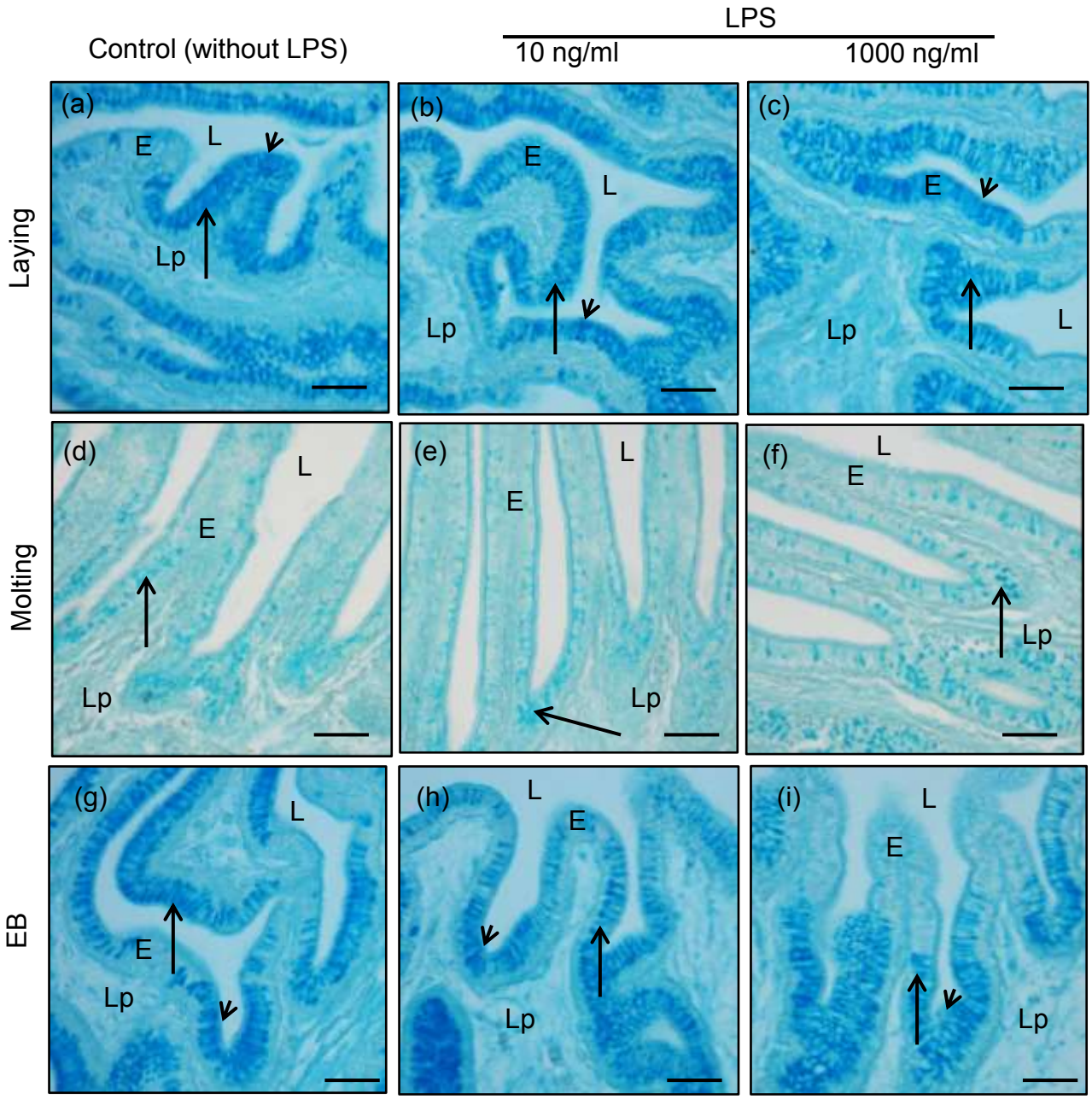

Figure 16. Localization of Alcian blue (AB) positive substances in the vagina of laying, molting, and EB groups treated with or without LPS. The mucosal tissues were well developed with formation of secondary folds and high epithelial cells lining the surface. The AB-positive substances are localized in the cells at the bottom of the secondary folds in laying groups (a). The density is reduced in the molting groups (d), and recovered in the EB groups (g). Treatment of the tissue with LPS does not affect the localization and density of the AB-positive substance in 10 ng LPS group (b, e, and h) and 100 ng LPS group (c, f, and i). LPS = lipopolysaccharide, EB = estradiol benzoate, E = mucosal epithelium, L = lumen of oviduct, Lp = lamina propria. Scale bars = 50 μm.

Chapter 4

TOLL-LIKE RECEPTOR SIGNALING FOR THE INDUCTION OF MUCIN EXPRESSION IN RESPONSE TO LIPOPOLYSACCHARIDE IN HEN VAGINA

INTRODUCTION

Mucin, one of the members forming mucosal barrier, plays essential role in the mucosal barrier functions. Lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, upregulated mucin expression in the oviduct of both laying and molting hens (Chapter 3). However, the intracellular mechanism by which mucin expression is upregulated by LPS in the oviductal mucosa remains unknown.

Toll-like receptors (TLRs) recognize pathogen associated molecular pattern and initiate innate immune response (Kogut et al., 2005). In chickens, genes of several types of TLR, namely, TLR1 to TLR5, TLR7, TLR15, and TLR21, have been identified (Temperley et al., 2008). It is reported that TLRs use adaptors of the Myeloid differentiation factor 88 (MyD88)- or Toll-interleukin 1 receptor (TIR) domain-containing adaptor-inducing IFN-β (TRIF) for activation of intracellular signal pathways (MyD88- and TRIF-dependent pathway, respectively). In mammals, TLR4 recognizes LPS, and interaction of TLR4 with LPS may activate both MyD88- and TRIF-dependent pathway in mammals (O'Neill and Bowie, 2007). In chickens, interaction of TLR4 and LPS may activate MyD88-dependent pathway, but not TRIF-dependent pathway, because adaptor protein TRIF-related adaptor molecule (TRAM) that bridges TLR4 and TRIF may lack (Keestra et al., 2013). At the end of TLR signal pathway, nuclear factor-κB (NFκB) and mitogen-activated protein kinase (MAPK) are activated. The NFκB is activated by breakdown of its inhibitor molecule (IκB), and the MAPK activates the activated protein 1 (AP1; cFos and cJun) (Kogut et al., 2012). The activated transcriptional factors, NFκB and AP1, are translocated into nucleus to initiate the transcriptions of the target genes (Kawai and Akira, 2010). The induction of mucin by LPS in the hen oviduct is probably mediated by TLR4 and its signal pathways, leading

to activation of transcriptional factors. We have identified expression of TLR4 in hen oviduct (Ozoe et al., 2009). However, it is unknown which signal pathway of TLR, namely MyD88-dependent or independent pathway is formed in the vaginal mucosa, and which transcriptional factors, NFκB or AP1, are responsible for mucin expression by LPS.

The aim of this Chapter was to determine the intracellular signaling molecules responsible for mucin induction by LPS, and the effects of molting and estrogen on their expression. Specific questions were asked to: 1) whether the expression of the TLR adaptor molecules and transcriptional factors are affected by the oviductal regression and estrogen, and 2) which transcriptional factors, NFκB and/or AP1, are involved in the mucin expression by LPS. In Experiment 1, the expressions of TLR4 and TLR adaptor molecules (MyD88 and TRIF), and transcriptional factors (NFκB, cFos, and cJun) were examined in laying and molting hens injected with or without estradiol. In Experiment 2, it was examined whether induction of mucin expression by LPS was suppressed by inhibitors of NFκB, IκB proteolysis, MAPK and AP1.

MATERIALS AND METHODS

Experimental birds and tissue preparation

White Leghorn hens, approximately 500-d-old and laying 4 or more eggs in a clutch, were used. They were kept in the same condition as Chapter 2. Induction of molting and daily injection of molting hens with 1 mg β-estradiol-benzoate (Sigma Co) for 7 days were also performed as described in Chapter 2 (molting and EB groups, respectively). The average weights of oviducts were 95.8 ± 1.6 g, 7.9 ± 0.8 g, and 32.5 ± 0.9 g in laying, molting and EB groups, respectively. The hens were euthanized under anesthesia with Somnopentyl (Kyoritsu Pharmaceutical Co., Tokyo, Japan) for sample collection. The birds were handled in accordance with the regulations of Hiroshima University Animal Research Committee.

In Experiment 1, the mucosal tissues of the vagina of laying hens (n = 5) were used for identification of the expression of TLR4, TLR adaptor molecules, and transcriptional factors in the vaginal mucosa. Then, the vaginal mucosa of laying,

molting and EB group hens were used to examine the effects of molting and estradiol on their expression (n = 5 each).

In Experiment 2, the vagina of laying hens was used (n = 5 in each LPS or inhibitor treatment analysis). The specimens of mucosa tissue were cultured as described previously (Chapter 3) and stimulated by LPS together with or without inhibitors of transcriptional factors, followed by mucin expression analysis. Inhibitors used in this study were ALLN (inhibitor of IκB proteolysis; EMD Chemicals Inc., San Diego, CA, USA), BAY-117085 (NFκB inhibitor; EMD Chemicals Inc.), U0126 (MAPK inhibitor; Promega Co., Madison, WI, USA), Transhinone IIA (AP-1 inhibitor; Santa Cruz Biotech., Inc., Santa Cruz, CA, USA). See Figure 17 for the TLR signaling molecules and inhibitors of them in chickens. The mucosal tissues of the vagina were cut into small pieces (approximately 1 x 1 x 5 mm) and placed in sterile tubes for culture (3 tissue pieces per tube) (Greiner Bio-one, Ltd., Tokyo, Japan) containing 4 ml culture medium with 100 ng/ml LPS, and 0 – 100 μM ALLN, BAY-117085, U0126 or Transhinone IIA. The culture medium was TCM-199 medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10% bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), 100 U/ml penicillin and 100 μg/ml Streptomycin (Cosmo Bio, Tokyo, Japan). Effects of LPS on mucin induction was confirmed by incubating tissues in the medium with LPS at concentrations of 10, 100, or 1000 ng/ml for 3 h, as described in Chapter 3. Stock solution of LPS was prepared by dissolving LPS (lipopolysaccharide from *Salmonella minnesota*; Invivogen, San Diego, CA, USA) in PBS at a concentration of 5 mg/ml. Incubation was performed in a CO_2 incubator at 37°C under a humidified atmosphere with 5% CO_2 and 95% air.

Quantitative RT-PCR analysis for expression of TLR4, adaptor molecules, transcriptional factors, and mucin

Expression of TLR4, adaptor molecules, and transcriptional factors in the vagina of hens in Experiment 1 and the mucin expression in cultured tissues in Experiment 2 were analyzed by RT-PCR and quantitative RT-PCR as described in Chapter 3. The total RNA samples were extracted and 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.

PCR was performed using Takara Ex Taq (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. Primers used in this study are shown in Table 2. The PCR mixture (25 μL) contained 0.5 μL cDNA, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1.25 U Takara Ex Taq, and 0.5 μM each primer. Amplification was performed in a PTC-100 Programmable Thermal Controller (MJ Research Inc., Waltham, MA, USA) under the following conditions: 94°C for 30 sec, then 34 cycles at 95°C for 30 sec to denature, 58°C for 60 sec to anneal, 72°C for 60 sec for extension. The PCR products were separated by electrophoresis on a 2% (w/v) agarose gel containing 0.04% (w/v) ethidium bromide.

Real-time PCR was performed using the Roche Light Cycler system (Roche Applied Science, Indianapolis, IN). The reaction mixture (20 μL) contained 3 μL of cDNA, 1 × SYBR Premix EX Taq (Takara Bio Inc.), and 0.5 μM of each primer. The mixture was placed into 20 μL capillaries (Roche Diagnostics GmbH, Mannheim, Germany). The cycle parameters for PCR reaction were at 95°C for 5 sec and 62°C for 20 sec. Real-time PCR data were analyzed by the $2^{-\Delta\Delta CT}$ method to calculate the relative level of mRNA in each sample using *RPS17* for the housekeeping gene as described in Chapter 3. A sample of the vaginal mucosa of a laying hen was used as the standard for comparison among laying, molting and EB group hens in Experiment 1. In Experiment 2, a sample of the vaginal mucosa incubated for 3 h without LPS was used as standard for the analysis of the effects of LPS on mucin expression. Also, the vagina mucosa incubated for 3 h with 100 ng/mL LPS was used as the standard to examine the effects of inhibitors of transcriptional factors on the LPS-induced mucin expression. The results were expressed as relative expression obtained from the ratio of the experimental samples and the standard sample.

Statistical analysis

The significance of the differences in the expressions of TLR4, MyD88, TRIF, NFκB, cFos, and cJun among laying, molting and EB groups in Experiment 1, and expression of mucin among different inhibitor doses groups in Experiment 2 was

examined by one-way ANOVA followed by Tukey's test or the Steel-Dwass test. Differences were considered significant at $P < 0.05$.

RESULTS

Experiment 1

RT-PCR analysis showed that TLR4 and its adaptor molecules of MyD88 and TRIF are expressed in the vaginal mucosa of laying groups. Expression of transcriptional factors, namely, NFκB1, cFos, and cJun were also confirmed (Figure 18).

Figure 19 shows expression of TLR4, MyD88, and TRIF in the vaginal mucosal tissue of laying, molting and EB groups. The relative expression levels of TLR4 and MyD88 were decreased to approximately 0.2 folds (Figure 19a, b), and that of TRIF was less than 0.5 folds (Figure 19c) in molting hens compared with laying hens. Their expression levels in the EB group were greater than in the molting group, although they were lower than laying group.

Figure 20 shows differences in the gene expression levels of transcriptional factors in the vaginal mucosa tissues of laying, molting and EB groups. The relative expression level of cFos, and cJun in the mucosal tissues were less than 0.6 folds in molting groups compared with laying groups (Figure 20b, c), whereas NFκB1 gene expression was not significantly different between laying and molting groups (Figure 20a). The expression level of cFos and cJun was greater in the vagina of EB group than that in the molting group (Figure 20b and c). The expression level of NFκB1 in the EB group was not different from molting group (Figure 20a), and was lower than laying group (Figure 20a).

Experiment 2

The effects of transcriptional factor inhibitors, namely, ALLN, BAY-117085, U0126, and Transhinone IIA on LPS-induced mucin mRNA expression in the vagina of laying hens are shown in Figure 21. The expression of mucin was increased by LPS

exposure in a dose dependent manner (Figure 21a). The mucin expression levels was significantly declined to approximately 0.5 folds by ALLN, BAY-117085, and U0126 at a dose of 10 μM, and further decreased at 100 μM dose (Figure 21b, c, and d). The expression level was also decreased by Transhinone IIA at 1 μM or more dose (Figure 21e).

Figure 22 shows the effect of single or simultaneous transcriptional factors inhibitor on LPS-induced mucin mRNA expression in the vagina of laying groups. The mucin expression levels was significantly declined to approximately 0.5 folds by BAY-117085 as well as Transhinone IIA at a dose of 10 μM, and further decreased by combination of both inhibitors (Figure 22a). The mucin expression levels was significantly declined to approximately 0.4 - 0.5 folds by ALLN, and U0126 at a dose of 10 μM, and further decreased by combination of both inhibitors (Figure 22b).

DISCUSSION

The results of this Chapter show that the intracellular signaling molecules involved in the LPS stimulation of mucin expression, and effect of molting and estrogen on their expressions. Major findings were: (1) expression of major TLR adaptor molecules (MyD88 and TRIF), and transcriptional factors (cFos and cJun) were significantly lower in molting hens compared with laying hens, and up-regulated by estrogen, and (2) mucin expression was up-regulated by LPS, whereas it was suppressed by inhibitor of transcriptional factors (IκB proteolysis, NFκB, MAPK, and AP-1 inhibitor).

It is accepted that there are two different TLR signaling pathways including MyD88-dependent and TRIF-dependent pathways (Kawai and Akira, 2010). The current results showed both MyD88 and TRIF were expressed in the vaginal mucosa, suggesting that both pathways for TLR signaling are formed in that tissue. Kogut et al. (2012) reported that infection by *Salmonella enteritidis* induced the attraction of both a MyD88-dependent and a TRIF-dependent TLR signaling pathways. It is reported in mammals that TLR4 is unique in that it utilizes both MyD88- and TRIF-dependent pathways (Kogut et al., 2012). However, in chicken, LPS failed to stimulate TLR4-TRAM-TRIF pathway (Keestra and van Putten, 2008; de Zoete et al., 2010), probably

due to absence of TRAM that bridges TLR4 and TRIF. Thus, although expression of MyD88 and TRIF was identified, mucin induction by LPS might be mediated by TLR4 and MyD88 pathway. The TRIF may function as an adaptor of other TLRs to recognize microbe components. MyD88 signaling pathway may link to activation of nuclear transcription factors including NFκB and AP1 (cFos and cJun) (Kogut et al., 2012; Keestra et al., 2013). The current results showed expression of NFκB, cFos and cJun in the vaginal mucosa, suggesting a possibility that these nuclear transcriptional factors may participate in the regulation of mucin expression by MyD88 signaling pathway.

The expression of TLR4 and MyD88 was declined in molting group and upregulated by EB. Thus, the ability to recognize LPS by TLR4 and signaling by MyD88 may be decreased in association with oviductal regression due to the decline of circulating estrogen level during molting. The cFos and cJun expression in molting hens was lower than laying hens, and recovered to the same level to laying hens by injection with EB. In contrast, NFκB1 expression was not significantly different between laying and molting hens, and the expression was not recovered by EB. In mammal, macrophages estradiol-17β prevents its nuclear translocation (Ghisletti et al., 2005). Thus, estrogen may not enhance the transcriptional activity by NFκB. It is likely that expression level of AP1 transcriptional factor declines during molting due to the less circulating estrogen level. However, the expression of NFκB may not be positively correlated with estrogen level.

The mucin expression was upregulated by LPS, supporting the results in Chapter 3. The effect of LPS on the mucin expression was suppressed by inhibitors for NFκB-dependent transcription, namely an inhibitor of IκB proteolysis (ALLN) and an inhibitor of NFκB nuclear translocation (BAY-117086). Also, the LPS effects was suppressed by inhibitors for transcriptional process by MAP kinase and AP1, namely an inhibitor of MAP kinase (U0126) and an inhibitor of AP1 (Transhinone IIA). These results suggest that induction of mucin expression by LPS is mediated by both NFκB-dependent and AP1-dependent pathways. Shen et al. (2008) reported that synergistic induction of mucin5AC in mixed infections by *Haemophilus influenzae* and *Streptococcus pneumoniae* is regulated by AP-1 in human colon and middle ear epithelial cells. It is also reported that mucin5AC hypersecretion was consistent with activation of AP-1 and NFκB signaling pathways in the downsream of epidermal growth factor and TLR4 in rat airway cells (Nie et al., 2012). More recently Yang et al. (2013)

reported that crotonaldehyde, a toxic α,β-unsaturated aldehyde, stimulated inflammatory response by enhancing IL-8 release from human airway epithelial cells by both NFκB and AP-1 pathways. Therefore, it is likely possible that a LPS stimulation activates both NFκB and AP-1 pathways for mucin induction in hen vagina as observed in this study. It is supported by the results that mucin expression induction in response to LPS was more suppressed by mixture of inhibitors for NFκB and AP-1 pathways (BAY-117086 plus Transhinone IIA, ALLN plus U0126) compared with single inhibitor effects.

In conclusion, MyD88-dependent and TRIF-dependent pathways for TLR signaling exist in the vagina. Expression of TLR adaptor molecules and transcriptional factors is reduced in molting phase compared with laying phase, probably due to the decline of estrogen level. Transcriptional factors of NFkB and AP-1 may participate in the induction of mucin expression by LPS. The mucin expression system in response to LPS may play a role in the mucosal barrier against infection.

ABSTRACT

The aim of this study was to determine the intracellular signaling molecules for mucin induction by lipopolysaccharide (LPS), and the effect of molting and estrogen on their expressions. Expression of TLR4, its adaptor molecules, and transcriptional factors in the vaginal mucosa of laying and molting hens treated with or without estradiol were examined by reverse transcription (RT)-PCR. Expression of mucin in the cultured mucosal tissue stimulated by LPS together with inhibitors of transcriptional factors was analyzed by quantitative RT-PCR. Expression of TLR4, its adaptor molecule, namely, myeloid differentiation factor 88 (MyD88) or toll-interleukin 1 receptor domain-containing adaptor-inducing IFN-β (TRIF), and transcriptional factors, namely, cFos, and cJun, were declined in molting hens compared with laying hens, and were upregulated by estradiol. In vagina of laying hens, mucin expression was upregulated by LPS, whereas it was suppressed by inhibitors of transcriptional factors, namely, ALLN (an inhibitor of IκB proteolysis), BAY-117085 (a NFκB inhibitor), U0126 (a mitogen-activated protein kinase [MAPK] inhibitor), and Transhinone IIA (an activated protein 1 [AP-1] inhibitor). These results suggest that MyD88-dependent pathway in the downstream of TLR4 and transcriptional factor of NFκB and AP-1 participate in the induction of mucin expression by LPS in the vaginal mucosa. These signaling functions may be declined during molting due to the decline of circulating estrogen level. Such mucin expression system may play roles in the mucosal barrier against infection in the vaginal mucosa.

Table 2. Primer sequences for *toll-like receptor 4 (TLR4),* TLR adaptor molecules, transcriptional factors, *mucin*, and *ribosomal protein S17 (RPS17).*

Target genes	Sequences 5' – 3'	Accession number References
TLR4	F: AGTCTGAAATTGCTGAGCTCAAAT	NM_001030693
	R: GCGACGTTAAGCCATGGAAG	Ozoe et al., 2009
MyD88	F: AAGTTGGGCCACGACTACCT	NM_001030962
	R:CAGAAAGGGTTGTTAAGCACTG	Kogut et al., 2012
TRIF	F:TCAGCCATTCTCCTGCCTCTTC	EF025853
	R:GGTCAGCAGAAGGATAAGGAAAGC	Kogut et al., 2012
NFKB1	F: GAAGGAATCGTACCGGGAACA	NM_205134
	R:CTCAGAGGGCCTTGTGACAGTAA	Chiang et al., 2009
cFos	F: ACCTCCACCTTCGTCTTCACCTAC	NM_205508
	R: TCGTTGCTAAGTCATCAGAACACG	Levy et al., 2003
cJun	F: GAGCCTACTTTCTACGAGGATGCC	NM_001031289
	R: GTTGCTGGACTGGATGATGAGC	Levy et al., 2003
Mucin	F: TCTTCCGCTACCCTGGGCTCTGTAA	AJ487010
	R: CTCATGCAGTTCTAGCAAGATACT	Smirnov et al., 2004
RPS17	F: AAGCTGCAGGAGGAGGAGAGG	NM_204217
	R: GGTTGGACAGGCTGCCGAAGT	Nii et al., 2011a

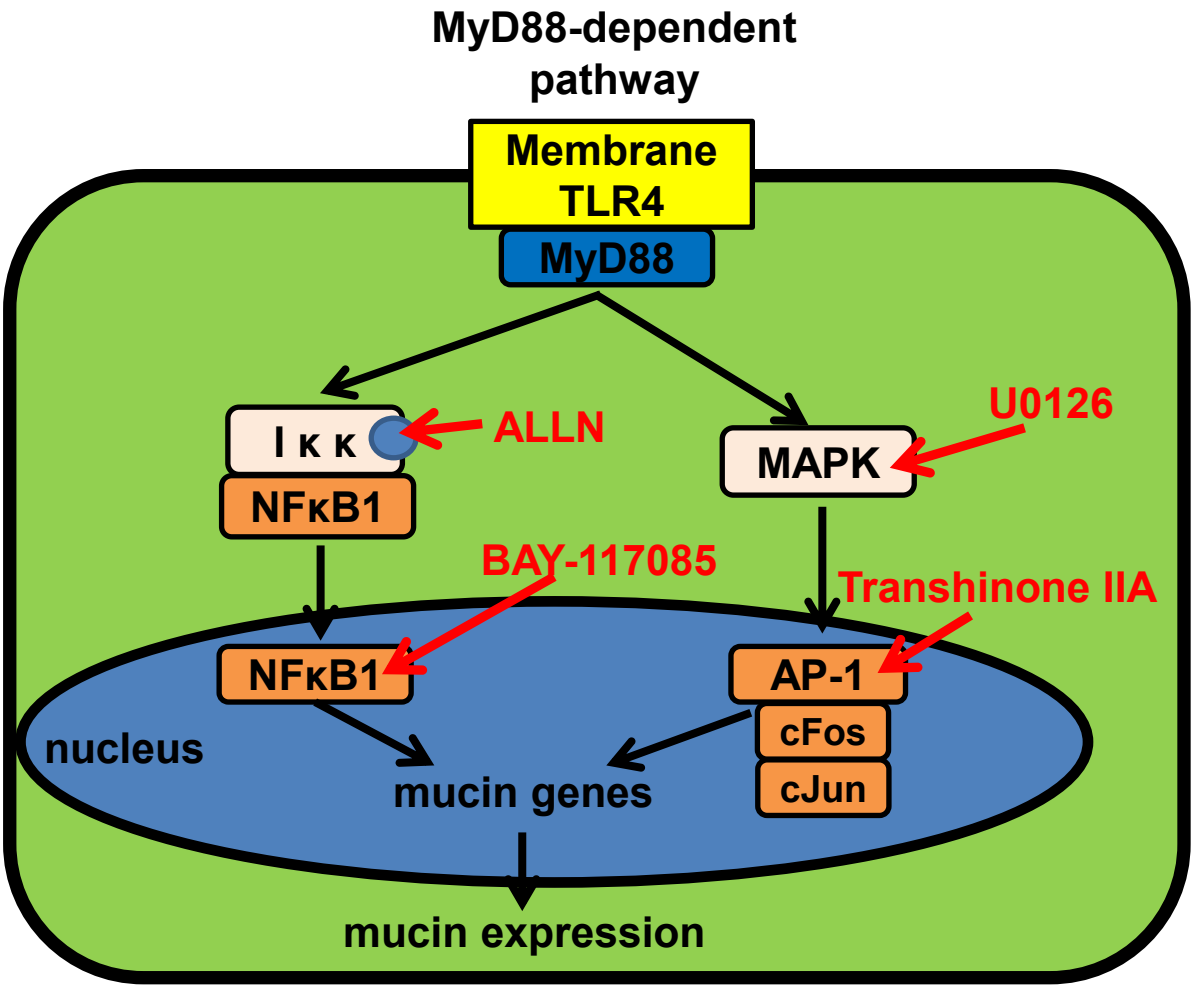

Figure 17. Representative diagrams describing the toll-like receptor (TLR) signaling molecules and inhibitors of them in chickens. Inhibitors are ALLN (inhibitor of IκB proteolysis), BAY-117085 (NFκB inhibitor), U0126 (MAPK inhibitor), and Transhinone IIA (AP-1 inhibitor). MyD88 = myeloid differentiation factor 88, MAPK = mitogen-activated protein kinase, Iκκ = inhihitor of κB kinase, NFκB = nuclear factor-κB, and AP1 = activated protein 1.

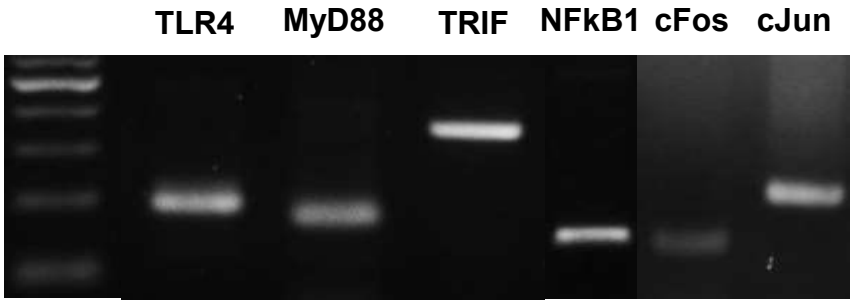

Figure 18. Reverse-transcription PCR analysis for the expression of toll-like receptor 4 (TLR4), TLR adaptor molecules (MyD88 and TRIF), and transcriptional factors (NFκB1, cFos, and cJun) in the vagina of laying group. MyD88 = myeloid differentiation factor 88, TRIF = toll-interleukin 1 receptor (TIR)-domain-containing adaptor-inducing interferon β, NFκB = nuclear factor-κB.

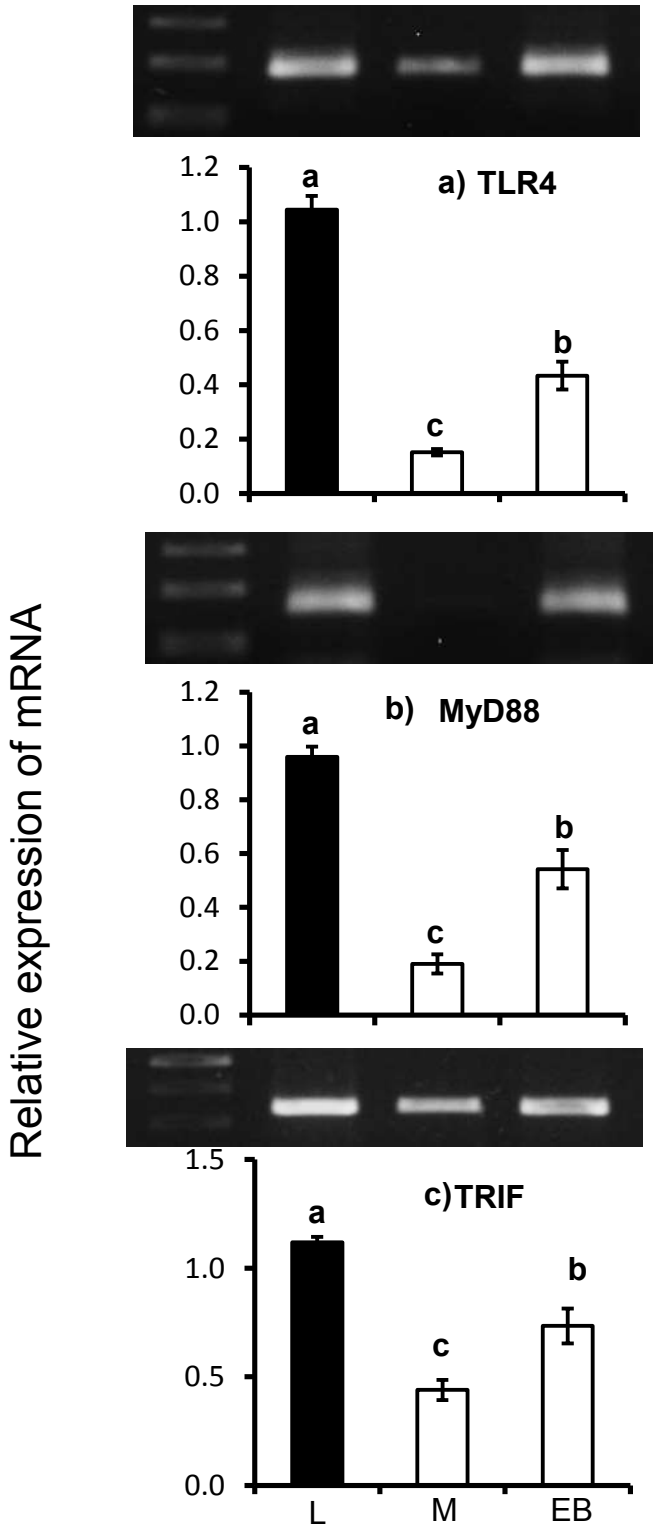

Figure 19. TLR4 and TLR adaptor molecules (MyD88 and TRIF) mRNA expression in the mucosal tissue of laying (L), molting (M), and EB groups in the vagina. Values are the mean ± SEM of relative expression (n = 5). EB = estradiol benzoate. [a,b,c] Values are significantly different among laying, molting, and EB groups (P<0.01). a) Steel-Dwass test; b) and c) Tukey's test. TLR = toll-like receptor, MyD88 = myeloid differentiation factor 88, TRIF = toll-interleukin 1 receptor (TIR)-domain-containing adaptor-inducing interferon β.

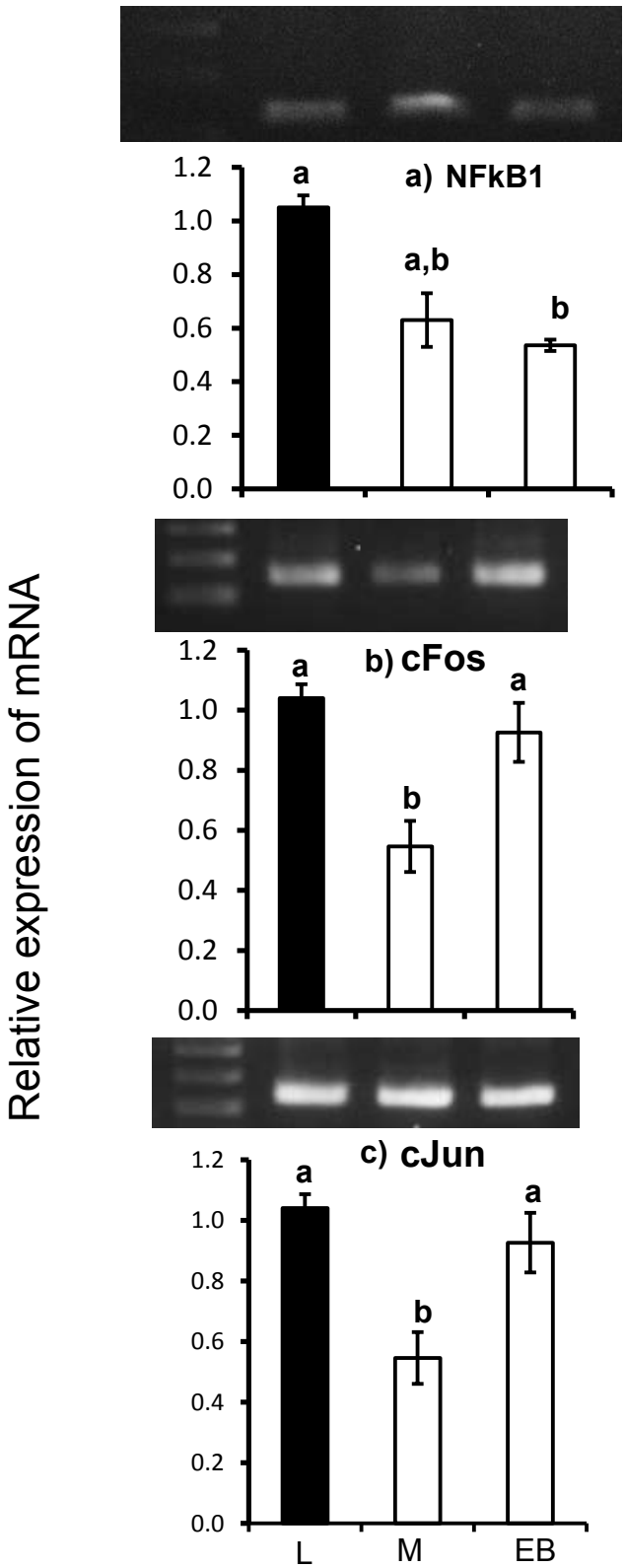

Figure 20. Transcriptional factors (NFκB1, cFos, and cJun) mRNA expression in the mucosal tissue of laying (L), molting (M), and EB groups in the vagina. Values are the mean ± SEM of relative expression (n = 5). EB = estradiol benzoate. [a,b,c] Values are significantly different among laying, molting, and EB groups (P<0.01). a) Steel-Dwass test; b) and c) Tukey's test. NFκB = nuclear factor-κB.

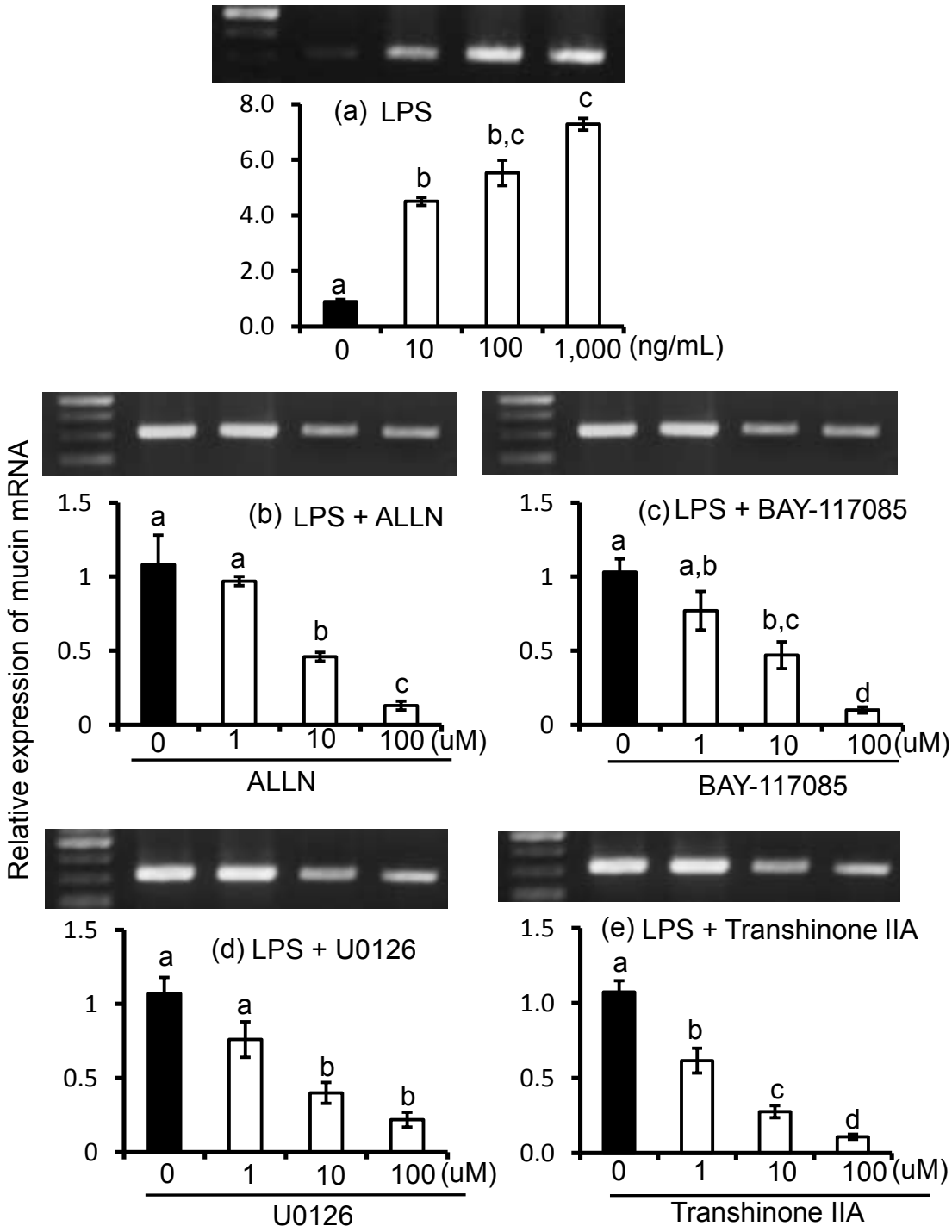

Figure 21. Effect of transcriptional factors inhibitors, namely, ALLN, BAY-117085, U0126, and Transhinone IIA on LPS-induced mucin mRNA expression in vagina of laying group.

(a). Mucosal tissues of vagina were cultured and treated with increasing concentrations of LPS (0, 10, 100, 1,000 ng/ml) for 3 h.

(b - e). The tissues were cultured and stimulated with 100 ng/ml LPS together with ALLN, BAY-117085, U0126, and Transhinone IIA.

Values are the mean ± SEM of relative expression (n=5). [a,b,c] Values are significantly different among different concentration of inhibitors. a), b), c), and e) Steel-Dwass test; d) Tukey's test. LPS = lipopolysaccharide.

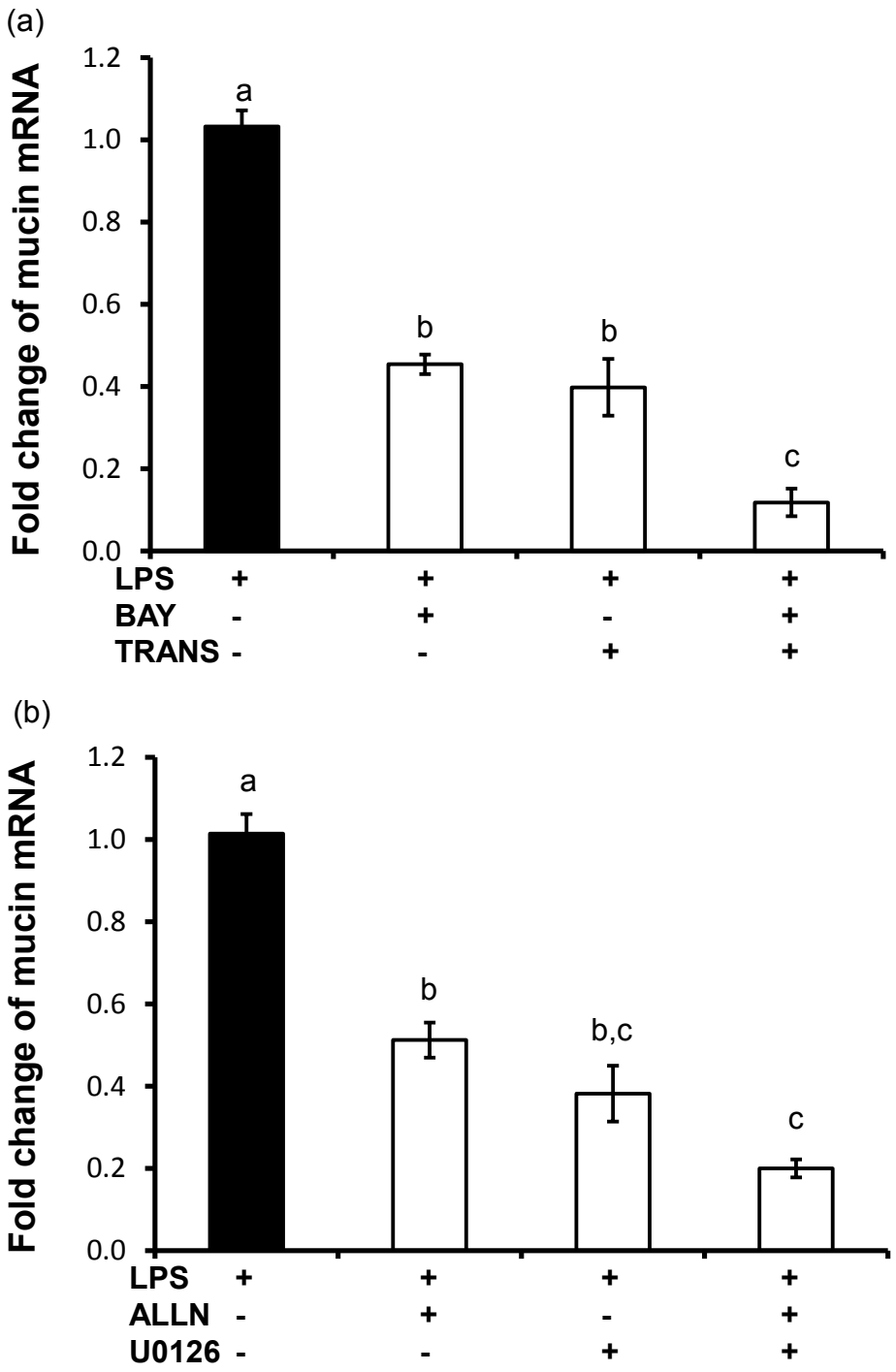

Figure 22. Effect of single or simultaneous transcriptional factors inhibitor on LPS-induced mucin mRNA expression in vagina of laying group.
(a) The tissues were cultured and stimulated with 100 ng/ml LPS for 3 h together with single and/or simultaneous of BAY-117085 and Transhinone IIA at concentration of 10 uM.
(b) The tissues were cultured and stimulated with 100 ng/ml LPS for 3 h together with single and/or simultaneous of ALLN and U0126 at concentration of 10 uM. Values are the mean ± SEM of relative expression (n=5). ^{a,b,c} Values are significantly different among different concentration of inhibitors. a) and b) Tukey's test. LPS = lipopolysaccharide.

Chapter 5

EXPRESSION OF TIGHT JUNCTION MOLECULE "CLAUDINS" IN THE LOWER OVIDUCTAL SEGMENTS AND THEIR CHANGES WITH EGG-LAYING PHASE AND GONADAL STEROID STIMULATION IN HENS

INTRODUCTION

In hens, microorganisms colonizing the cloaca may ascend the oviduct through the vagina and uterus. Mucosal barrier systems formed by epithelial cell junctions, mucin layer, and leukocyte activity play primary roles to prevent their infection. Tight junctions (TJ) of mucosal epithelium are responsible for a paracellular barrier that protects underlying tissue against the most toxic materials or pathogens (Van Italie and Anderson, 2006; Forster, 2008; Goto and Kiyono, 2012). Members of the claudin protein family form the main components of TJ and each family member laterally interacts with another claudin protein located in the adjacent cell in a homotypic or heterotypic manner (Tsukita et al., 2001; Krause et al., 2008). They assemble in the plasma membrane together with other proteins of the TJ complex, such as occludin and tricellulin (Forster, 2008; Will et al., 2008). Currently, nucleotide sequences of 3 *claudins* (*claudin-1, -3,* and -5) have been reported and 15 *claudins* (*claudin-2, -4, -8, -10-12, 14-20,* and *22-23*) are predicted in the database of the National Center of Biotechnology Information (NCBI, 2012). *Claudin-1* was found to be present in high-resistance epithelia and crucial for the epidermal barrier. *Claudin-3* was found to be present in the tighter segment of the nephron, and *claudin-5* was shown to constitute TJ strands in endothelial cells (Gonzales-Mariscal et al., 2003; Turksen and Troy, 2004). Thus, *claudin-1, -3*, and *-5* are likely to be expressed to form tight junctions in epithelial tissues. In birds, Park et al. (2011) reported that the level of *claudin-1* mRNA in immature testes was higher than that of adult testes of pheasant (*Phasianus colchicus*). Ozden et al. (2010) showed that *claudin-3, -5*, and *-16* expression in the intestinal epithelium was higher in 2-day-old chicks than in 20-day

embryo. Thus, claudin synthesis may be affected by physiological and environmental factors.

Previous reports suggested that oviduct was more susceptible in molting hens than laying hens as described previously (Chapter 2, 3; Golden et al., 2008). If the expression of claudins in the oviductal epithelium declines, mucosal barrier functions may be weakened, leading to increased susceptibility to pathogenic agents. It is reported that proinflammatory cytokines such as *TNFα* and *IFNɣ* downregulated the expression of claudins and increased paracellular permeability in the epithelium (Baker et al., 2008; Li et al., 2008; Mazzon and Cuzzocrea, 2008). In Chapters 2 and 3, it was shown that mucin expression was reduced in the oviduct during molting phase, which may result in the reduction of mucin barrier on the tissue surface. 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 oviduct is up-regulated by this steroid (Oka and Schimke, 1969).

The goal of this study was to determine the differences in the expression of claudins in the lower oviductal segments (the vagina, uterus, and isthmus) at different egg-laying phases, namely, laying and molting, and the role of gonadal steroid in their expression in hens. Specific questions were whether: (1) expression of *claudin-1, -3*, and *-5*, (2) expression of lipopolysaccharide-induced *TNFα* factor (*LITAF*) and *IFNɣ*, and (3) permeability of FITC-dextran within mucosal epithelium in the lower oviductal segments were different between laying and molting phases and changed by estrogen stimulation.

MATERIALS AND METHODS

Experimental birds

Healthy White Leghorn laying and molting hens of approximately 500-days-old were kept as described in Chapter 2. The laying hens regularly laying 4 or more eggs in a clutch were provided with feed and water ad libitum. Molting was induced by feed regulation (25 g/d), and a proportion of the molting hens were intramuscularly injected daily with 1 mg of β-estradiol benzoate (Sigma-Aldrich Co., St. Louis, MO, USA; EB

group) or 100 μL of sesame oil (control group) for 7 days as described in Chapter 2. The birds were handled in accordance with the regulations of Hiroshima University for animal experiments.

Analysis of claudin and cytokine expression

The isthmus, uterus, and vagina of the laying (6 h after oviposition), molting, EB, and control groups were collected after euthanization under anesthesia with Somnopentyl (Kyoritsu Seiyaku Inc., Tokyo, Japan) (n = 5 in each group).

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

Total RNA was extracted from the mucosal tissues of the isthmus, uterus, and vagina of each experimental bird using Sepasol RNA I Super (Nacalai Tesque Inc., Kyoto, Japan). The RNA samples were reverse-transcribed using ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan) according to the manufacturer's instructions as described in Chapter 2.

PCR was performed using Takara Ex *Taq* (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. The primers for *claudins*, *LITAF*, *IFNγ*, and *RPS17* used in this study are shown in Table 3. The PCR mixture (25 μL) contained 0.5 μL of cDNA, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 1.25 U Takara Ex *Taq*, 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 sec, and then 33 cycles at 95°C for 30 sec for denaturing, 65°C for 30 sec for annealing, and 72°C for 45 sec for extension. The PCR products were separated by electrophoresis on a 2% (w/v) agarose gel containing 0.04% (w/v) ethidium bromide.

Real-time PCR was performed using the Roche Light Cycler system (Roche Applied Science, Indianapolis, IN). The 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 sec and 62°C for 20 sec. Real-time PCR data were analyzed by the $2^{-\Delta\Delta CT}$ method to calculate the relative level of mRNA in each sample using *RPS17* for the housekeeping gene (Livak and

Schmittgen, 2001). 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 groups, and samples from a non-treated molting hen were used as standard for comparison between EB and control groups. The results were expressed as relative expression obtained from the ratio between the experimental samples and the standard sample.

Claudin-1 immunohistochemistry

Tissue samples of isthmus, uterus, and vagina of each bird were fixed in 10% (v/v) formalin-PBS, dehydrated, and embedded in paraffin. Their sections (4 μm in thickness) were air-dried on slides, and used for hematoxylin and eosin (HE) staining and immunohistochemistry for claudin-1. For immunohistochemistry, sections were deparaffinized and autoclaved in 10 mM sodium citrate, pH 6.0, for 1 min. After washing in PBS (3 times for 5 min each), the sections were incubated with 5% (v/v) normal horse serum for 30 min, followed by overnight incubation with goat anti-claudin-1 polyclonal antibody (Santa Cruz Biotech., Inc., Santa Cruz, CA, USA) diluted at a concentration of 2 μg/ml in PBS. After washing in PBS, the sections were incubated with biotinylated donkey anti-goat IgG (Santa Cruz Biotech., Inc.) for 1 h. The immunoreaction products were detected by incubating with avidin-biotin-peroxidase complex (Vector Lab. Inc., Burlingame, CA) for 1 h, followed by color development using a reaction mixture of 0.02% (w/v) 3',3'-diaminobenzidine-tetrahydrochloride and 0.005% (v/v) H_2O_2 in 0.05 M Tris-HCl, pH 7.6 (DAB-H_2O_2 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.

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 using Somnopentyl (Kyoritsu Seiyaku Inc.). Five mL of FITC-dextran solution was injected using a syringe with a 23G needle into the uterus, and recovered by closing the abdominal wall. The solution was prepared by dissolving FITC-dextran (3000 –

5,000 molecular weight; Sigma-Aldrich Co.) in PBS at a concentration of 250 μM. After 3 h post-injection, the birds were euthanized under anesthesia with Somnopentyl (Kyoritsu Seiyaku Inc.), and the uterus tissue was embedded in cryo-embedding medium (Tissue-Tek O.C.T. Compound, Sakura Finetek Inc., Torrance, CA, USA) and snap-frozen in dry ice-isopentane mixture. Kabashima et al. (2002) reported invasion of FITC-dextran in the mouse intestinal mucosa by 3 h. Cryostat sections were air-dried on slides, fixed with 10% (v/v) formalin in PBS, and covered with glycerol. The sections were examined under a fluorescence microscope (Nikon Eclipse E600, 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. Uterus tissue of non-injected molting hens was also observed identically to examine the specificity of FITC fluorescence.

Statistical analysis

The relative expressions of *claudins, LITAF,* and *IFNγ* are expressed as the 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 *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

Figure 23 shows the expression of *claudin-1, -3*, and *-5* in the vagina, uterus, and isthmus of laying and molting groups. 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 group compared with laying group within each corresponding segment. The expression level of *claudin-3* was significantly reduced in molting group compared with laying group; namely, the levels of *claudin-3* expression in the three segments were approximately 0.9 to 1.2 in laying group, whereas they were 0.2 to 0.4 in molting group. 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 and 0.2 to 0.3 in the molting group.

The expression levels of *claudin-1, -3*, and *-5* in the vagina, uterus, and isthmus of control and EB groups are shown in Figure 24. The expression level was significantly greater in the EB group than in the control group at approximately 15 to 20-fold for *claudin-1*, 10 to 30-fold for *claudin-3*, and 7 to 12-fold for *claudin-5*.

Figure 25 shows the expression of *LITAF* and *IFNɣ* in the vagina, uterus, and isthmus of laying and molting groups. The relative expression levels of *LITAF* and *IFNɣ* were significantly higher in molting group than in laying group within each corresponding segment (Figure 25).

Figure 26 shows the localization of immunoreactive claudin-1 in the vagina, uterus, and isthmus. The mucosal surface of the vagina, uterus, and isthmus was lined by ciliated pseudostratified epithelium in both laying (Figure 26a-c) and molting groups (Figure 26d-f). Tubular glands were well developed in the lamina propria of uterus and isthmus (Figure 26b-c). The surface epithelium of each segment was thinner and tubular glands in the uterus and isthmus were involuted in molting group (Figure 26d-f). In the EB group, the surface epithelium became thicker than that in the control group (Figure 26g-i for control group; Figure 26j-l for EB group), and tubular glands appeared in the lamina propria of the uterus and isthmus (Figure 26k-l). In laying group, the claudin-1 immunoreaction products were identified on the apical and lateral region of the mucosal epithelial cells in each segment (Figure 26a-c). In molting group, granule-like immunoreaction products were observed in the mucosal epithelium of the isthmus (Figure 26f), whereas the products were negligible in the vagina and uterus (Figure 26d-e). In EB group, the immunoreaction products were identified in the lateral region of the mucosal epithelium in the vagina (Figure 26g), and the apical region of mucosal epithelium in the uterus and isthmus (Figure 26h-i). In contrast, in the control group, granule-like immunoreaction products were observed in the epithelium of the isthmus (Figure 26l), whereas they were negligible in those of the vagina and uterus (Figure 26j-k).

Figure 27 shows mucosal infiltration of FITC-dextran in the uterus of laying, molting, and EB groups. Fluorescence signal was identified in the apical region of mucosal epithelium in molting group (Figure 27b), whereas it was negligible in the laying group (Figure 27a) and the EB group (Figure 27c). Autofluorescence of endogenous substances appeared in the lamina propria of molting and EB groups

(Figure 27b-c). It was also observed in the uterus of molting group hens that were not injected with FITC-dextran (Figure 27d).

DISCUSSION

The current results show that the expression of claudins, molecules that form tight junctions, is affected by the egg-laying phase and estrogen in the lower oviductal segments (vagina, uterus, and isthmus) in hens. Significant findings were: (1) expression of *claudin-1, -3,* and *-5* genes and claudin-1 protein in the lower oviductal segments was higher in laying groups than in molting groups, (2) their expression was upregulated by estradiol, (3) expression of *LITAF* and *IFNγ* genes was higher in molting groups than in laying groups, and (4) more FITC-dextran infiltrated into the intercellular space of the uterus mucosal epithelium in molting group than in laying group and EB group.

Tight junctions create the variable barrier regulating paracellular movement of molecules through the epithelium and maintaining tissue homeostasis (Gonzales-Mariscal et al., 2003; Itoh and Bissel, 2003; Angelow et al., 2008). Claudins are a family of tight junction membrane proteins expressed in various epithelial tissues (Gonzales-Mariscal et al., 2003). The gene expression of *claudin-1, -3*, and *-5* suggests that tight junction is formed in the mucosa of the lower oviductal segments in hens. In addition, immunohistochemistry results confirmed the presence of claudin-1 in the mucosal epithelium of these oviductal segments.

The current results indicate that the expression of *claudin-1, -3,* and *-5* mRNA and immunoreactive claudin-1 was reduced in the oviductal mucosa of molting hens. The interpretation was that tight junctional functions such as acting as a barrier to invading agents and regulation of paracellular permeability may be disrupted in molting hens. FITC-dextran injected into the uterus lumen invaded into the paracellular space of mucosal epithelium in molting hens but not in laying hens, suggesting that the barrier function of mucosal epithelium was weakened in molting hens. Thus, it is likely that *claudin-1, -3,* and *-5* expression decreases in association with increased permeability in the regressed oviduct of molting hens. It may 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 (Moore and Holt, 2006; Dunkley et al., 2007; Golden et al., 2008). This may be due not only to 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.

The current study also showed that the expression of *LITAF* and *IFNɣ* genes was higher in molting hens than in laying hens. The expression of proinflammatory cytokines such as *IL1β, IL6*, and *IFNɣ* was higher in the oviduct of molting hens than in laying hens (Sundaresan et al., 2008; Nii et al., 2011). The level of *LITAF*, one of the transcription factors regulating the expression of *TNFα*, was also higher in the oviduct of molting hens (Sundaresan et al., 2007). It has been reported that *TNFα* and *IFNɣ* disrupt epithelial barrier function by influencing tight junctional functions (Bruewer et al., 2003; Baker et al., 2008; Li et al., 2008; Mazzon and Cuzzocrea, 2008). These proinflammatory cytokines downregulated *claudin-1* in the salivary epithelium (Baker et al., 2008). Thus, proinflammatory cytokines, *IFNɣ* and *TNFα*-like molecule, may participate in the reduction of *claudin* expression that increases paracellular permeability of epithelium in the regressed oviduct of molting hens.

Injection of molting birds with estradiol upregulated the expression of *claudin-1, -3*, and *-5* mRNA and claudin-1 protein in the mucosal epithelial cells. Estradiol-17β had no significant effect on the expression of *claudin-4, zonula-occludens-1,* or *E-cadherin*, although it modulated expression of *occludin* in the human cervical epithelial cells (Zeng et al., 2004; Gorodeski, 2007). In contrast, treatment of murine endothelial cells expressing estrogen receptors with estradiol-17β led to an increase in transendothelial electric resistance and upregulation of *claudin-5* (Burek et al., 2010). Thus, the effects of estrogen on the expression of *claudins* may differ among different *claudins* and cell types. In hen oviduct, it is likely that estrogen upregulated *claudin-1, -3,* and *-5* expression. This may 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 (Park et al., 2011). *Claudin-1* and *-3* expression was also identified in the developing

embryo of chicks (Haworth et al., 2005; Simard et al., 2006). Ozden et al. (2010) localized claudin-3, -5, and -16 proteins in the intestinal epithelium during the 3rd week of chick embryonic development. They suggested that, in addition to the barrier and fence functions within the tight junction, these *claudins* may have an additional role in the differentiation and/or physiological function in chick intestine. Blanchard et al. (2006) and Blanchard et al. (2009) reported that *claudin-1, -3*, and *-4* are differentially expressed in the mouse mammary gland during pregnancy, lactation, and involution, suggesting that different *claudin* family members may have functional importance at different times during mammary gland development. Thus, *claudins* may be expressed in various developing tissues to regulate their morphogenesis, growth, and differentiation (Gonzales-Mariscal et al., 2003; Turksen and Troy, 2004). The current study concluded that estrogen upregulated the expression of *claudin-1, -3*, and *-5* in the lower oviductal segments in association with the development of these tissues. The surface epithelium also became thicker by injection with estrogen in molting hens in this study. The expressed *claudins* may play roles not only in barrier formation but also in the development and differentiation of oviductal mucosal epithelium.

In conclusion, the results of this study have demonstrated that *claudin-1, -3*, and *-5* were expressed in the mucosal tissue of lower oviductal segment in laying hens. Their expression declined in molting hens and was upregulated by estrogen. The barrier functions of the mucosal epithelium may be disrupted due to reduction of claudin expression, which may lead the mucosal tissue to become more susceptible during the molting phase.

ABSTRACT

Tight junction in the mucosal epithelium plays essential roles as a mucosal barrier to prevent the invasion of microbes into the mucosal tissue of hen oviduct. The aim of this study was to determine the effects of egg-laying phase and gonadal steroid on the expression of tight junction molecule "claudins" in the lower oviductal segments in hens. White Leghorn laying and molting hens were used. A proportion of the molting hens (n = 5 in each group) were injected with sesame oil (control) or estradiol benzoate (EB). 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 *IFNɣ* was analyzed by quantitative RT-PCR, and localization of claudin-1 was examined by immunohistochemistry. Permeability in the mucosal epithelium was examined by intrauterine injection of fluorescein isothiocyanate (FITC)-dextran. Expression of *claudin-1, -3,* and *-5* genes and density of claudin-1 protein in the lower oviductal segments were higher in laying group than in molting group ($P<0.01$), and their expression was upregulated by EB ($P<0.01$). Expression of *LITAF* and *IFNɣ* genes was higher in molting group than in laying group. More FITC-dextran infiltrated into the intercellular space of the uterus mucosal epithelium in molting group than in laying group and EB groups. These results suggest that barrier functions of the mucosal epithelium at the lower oviductal segments may be disrupted due to reduction of claudin expression in molting hens, and it may lead the mucosal tissue to become more susceptible during the molting phase.

Table 3. Primer sequences for *claudins,* lipopolysaccharide-induced TNFα factor (*LITAF), IFNɣ,* and ribosomal protein S17 *(RPS17).*

Target genes	Sequences 5' – 3'	Accession number References
Claudin-1	F: GAC TCG CTG CTT AAG CTG GA	AY750897
	R: AAA TCT GGT GTT AAC GGG TG	Park et al., 2011
Claudin-3	F: AGC CCT CCA TCT CAG CAG	NM_204202
	R: TTC TCC GCC AGA CTC TCC	Ozden et al., 2010
Claudin-5	F: GTC CCG CTC TGC TGG TTC	NM_204201
	R: CCC TAT CTC CCG CTT CTG G	Ozden et al., 2010
LITAF	F: TGT GTA TGT GCA GCA ACC CGT AGT	AY765397
	R: GGC ATT GCA ATT TGG ACA GAA GT	Das et al., 2009
IFNɣ	F: ACT GAG CCA GAT TGT TTC GAT GT	X99774
	R: TGC CAT TAG CAA TTG CAT CTC CT	Sato et al., 2009
RPS17	F: AAG CTG CAG GAG GAG GAG AGG	NM_204217
	R: GGT TGG ACA GGC TGC CGA AGT	Nii et al., 2011

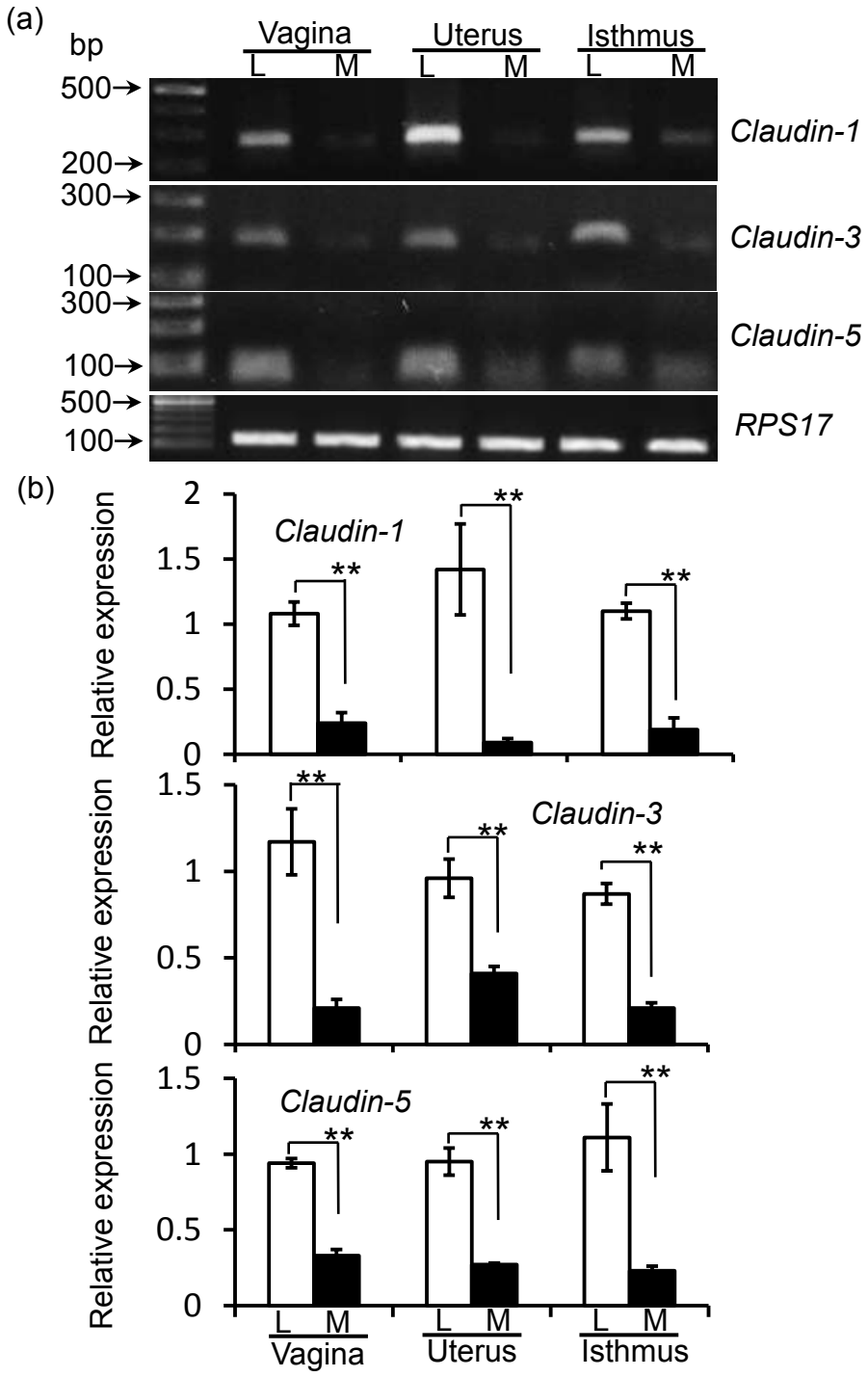

Figure 23. Expression of *claudin-1, -3,* and *-5* in the vagina, uterus, and isthmus of laying and molting groups. (a) PCR products of each claudin separated on agarose gel. (b) Relative expression of each claudin in laying and molting groups examined using a sample of laying group as standard within each oviduct segment. Values are the mean ± SEM of relative expression (n=5). ** Values are significantly different between laying and molting groups (P<0.01; *t*-test). L = laying group, M = molting group.

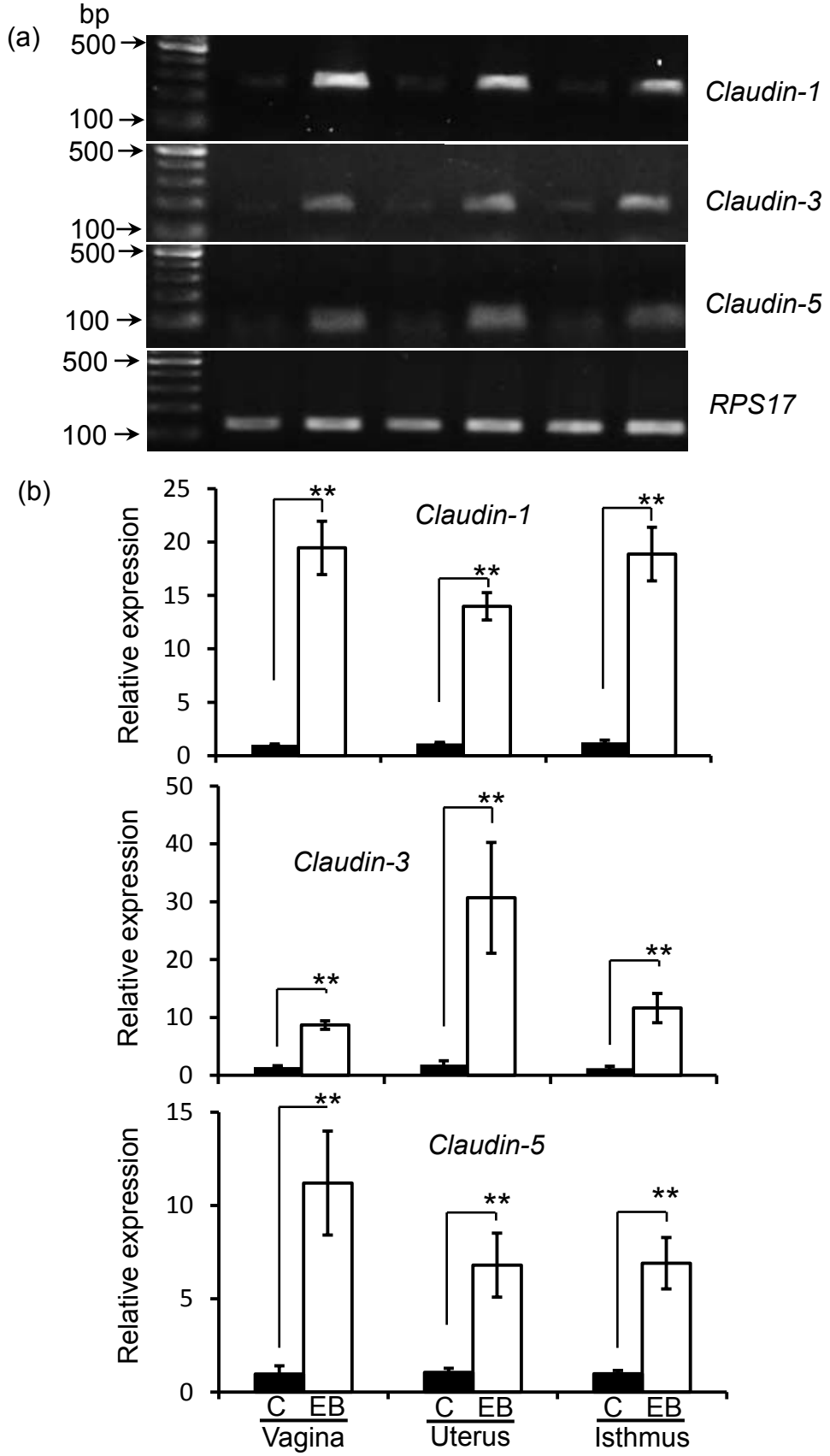

Figure 24. Expression levels of *claudin-1, -3,* and *-5* in the vagina, uterus, and isthmus of control (C) and EB groups. (a) PCR products of each claudin separated on agarose gel. (b) Relative expression of each claudin in the control group and the EB group examined using a sample of control bird as standard within each oviductal segment. Values are the mean ± SEM of relative expression (n=5). ** Values are significantly different between molting hens in control and EB groups (P<0.01; *t*-test).

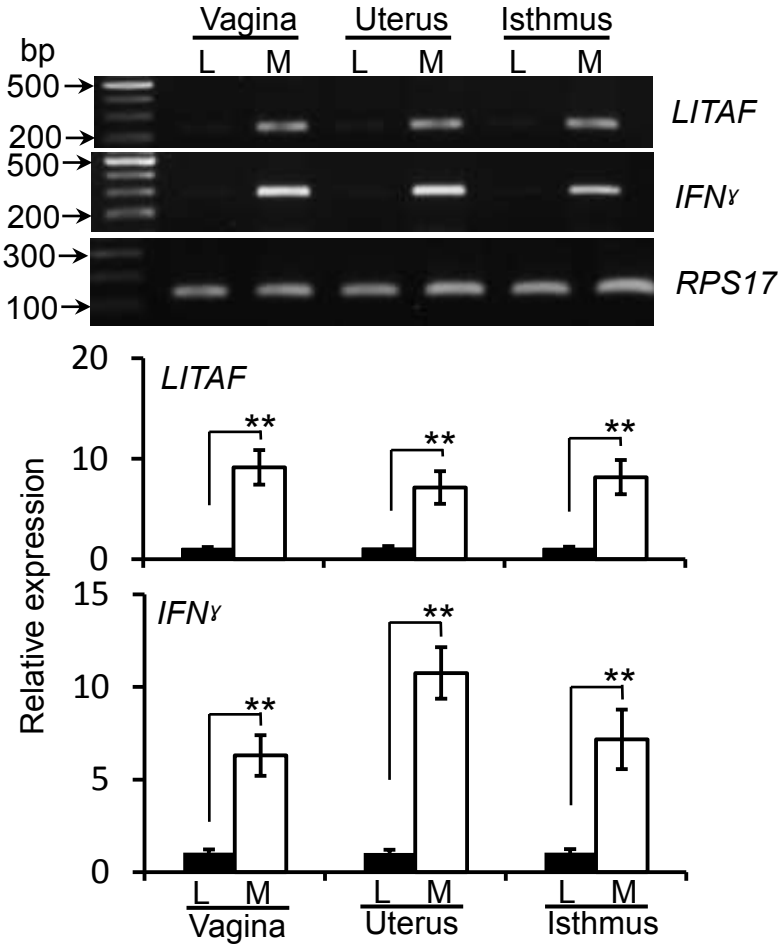

Figure 25. Expression of lipopolysaccharide-induced *TNFα* factor (*LITAF*) and *IFNɣ* in the vagina, uterus, and isthmus of laying and molting groups. Values are the mean ± SEM of relative expression using a sample of laying groups as standard within each oviductal segment (n=5). ** Values are significantly different between laying and molting groups (P<0.01; *t*-test). L = laying group, M = molting group.

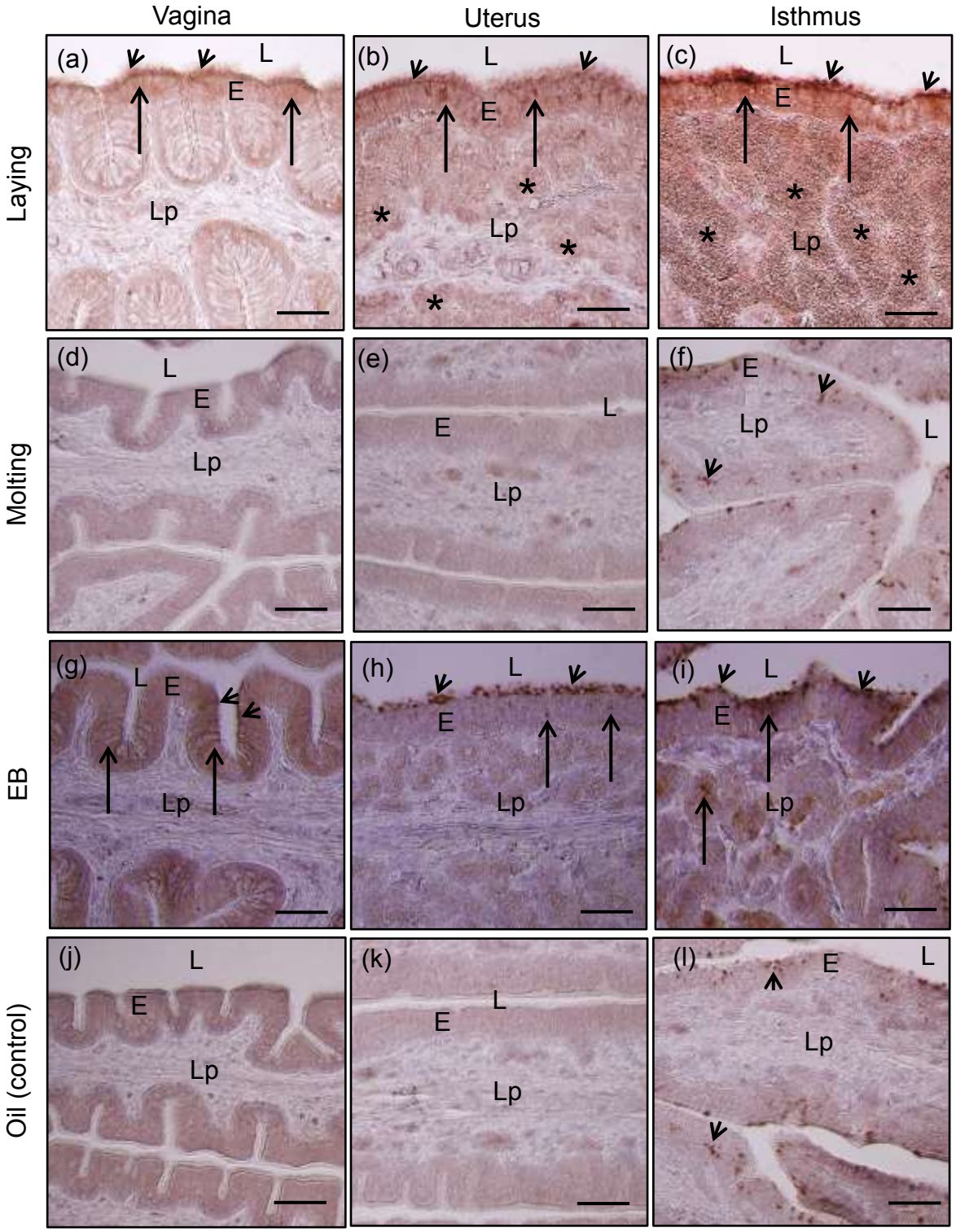

Figure 26. Localization of immunoreactive claudin-1 in the vagina, uterus, and isthmus of laying, molting, EB, and control groups. (a-c) Laying groups. Note that immunoreaction products are localized in the apical (short arrows) and lateral region (long arrows) of the mucosal epithelial cells in the three segments. (d-f) Molting groups. Immunoreaction products are negligible in the vagina and uterus (d-e), whereas granule-like products are identified in the mucosal epithelium of the isthmus (f, arrows). (g-i) EB groups. Immunoreaction products are identified in the apical (short arrows) and lateral regions (long arrows) of the mucosal epithelium in each segment. (j-l) control group. Immunoreaction products are identified only in the isthmus (l, arrows). E = mucosal epithelium, L = lumen of oviduct, Lp = lamina propria, asterisk = tubular glands. Scale bars = 50 μm.

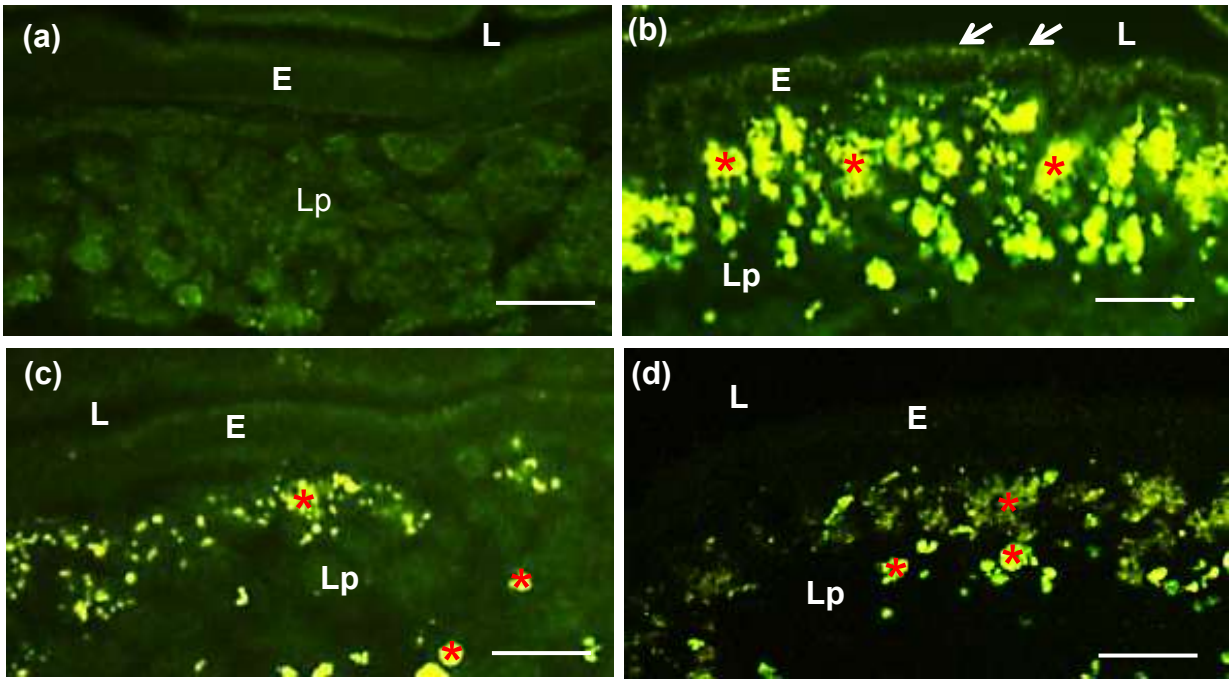

Figure 27. Infiltration of FITC-dextran in the mucosal epithelium of uterus of laying, molting, and EB groups. FITC-dextran was injected into the uterus lumen, and the sections of mucosal tissues were examined under a fluorescence microscope. Fluorescence signal is identified in the apical region of mucosal epithelium in molting group (b, arrows), whereas it is negligible in the laying (a) and EB groups (c). Autofluorescence of endogenous substances appears in the lamina propria of molting and EB groups (asterisks). Presence of autofluorescence is confirmed in the uterus of molting groups that were not injected with FITC-dextran (d). E = mucosal epithelium, L = lumen of oviduct, Lp = lamina propria. Scale bars = 50 μm.

Chapter 6

GENERAL DISCUSSION

Production of safe or hygienic eggs is important issues for the poultry industry. Infection in the oviduct by pathogenic organisms such as *Salmonella* results in the egg contamination and causes food-borne illness of human (Ricke et al., 2013). Almost 25 years ago the Centers for Diseases Control (CDC) estimated that in the case of salmonellosis, 170,000 patients visited a physician, 16,400 cases required hospitalization, and 600 cases resulted in death in the USA (Cohen and Tauxe, 1986). By the late 1990s, it was estimated that 500,000 cases of human illness were annually attributable to *Salmonella enteritidis* (Kaiser and Lamont, 2001). More recent information indicates that *Salmonella enteritidis* is the second most frequently isolated serotype of *Salmonella* from human illness in United States, and by 2006 it was responsible for almost 17% of reported cases of salmonellosis (Howard et al., 2012).

In general, mucosal barrier systems formed by mucus gel, epithelial cell junctional structures, and leukocyte activity, play important role to prevent infection by pathogenic agents in mucosal tissues. Mucins have the ability to form a physical barrier and act as adhesion decoys to invading agents (Linden et al., 2008a), and they may prevent pathogen penetrance by inhibiting bacterial adhesion to the mucosal epithelium surface (Berry et al., 2002). Mucins either have direct antimicrobial activity or carry other antimicrobial molecules (Linden et al., 2008b). Cell surface mucins may also initiate intracellular signaling in response to bacteria, and thus they have both a barrier and reporting function on the apical surface of mucosal epithelial cells (Linden et al., 2008a). Tight junctions of mucosal epithelium function as a paracellular barrier that protects underlying tissue from invasion of the most toxic materials or pathogens (Van Italie and Anderson, 2006; Forster, 2008; Goto and Kiyono, 2012). They create the variable barrier regulating paracellular movement of molecules through the epithelium and maintaining tissue homeostasis (Gonzales-Mariscal et al., 2003; Itoh and Bissel, 2003; Angelow et al., 2008). If microorganisms cross an epithelial barrier and begin to replicate in the mucosal tissues, phagocytic cells including the monocytes or macrophages, or polymorphonuclear leukocytes (PMNs) recognize, ingest, and destroy

them (Murphy et al., 2007; Macia et al., 2012). Thus, it is of great importance to identify the mechanism by which mucin is synthesized and epithelial tight junctions are formed in the oviduct of hens to prevent infection of this organ and contamination of eggs by pathogenic agents.

The current study showed that mucins were expressed in mucosal tissue of the lower oviductal segment in laying hens. The expression declined in molting hens, but was upregulated by estrogen in association with epithelial differentiation and development in the vagina, uterus, and isthmus. The reduction of mucin synthesis in the molting hens may result in the decline of mucosal barrier functions, leading to greater susceptibility to pathogens than in the laying hens (Chapter 2).

Bacterial LPS stimulated mucin expression in those epithelial cells of both laying and molting hens, which may enhance mucosal barrier function and play role in preventing infections (Chapter 3). The current results also showed the existence of TLR4 and MyD88 signal pathway and NFκB and AP-1 transcriptional pathway. Transcriptional factors of NFkB and AP-1 in the downstream of TLR4 may participate in the induction of mucin expression by LPS since their inhibitors suppressed that mucin expression. The TRIF, another TLR adaptor, identified in the vaginal mucosa may play roles as an adaptor molecule for TLRs other than TLR4 (Chapter 4). Further studies are necessary to determine whether recognition of microbe components other than LPS by different TLRs that activate nuclear transcriptional factors through TRIF dependent pathway induces mucin expression.

Expression of *mucin* mRNA and the density of AB-positive substances were reduced (Chapter 2), mucin expression after LPS treatment was lower (Chapter 3), and expression of TLR4 and MyD88 were also lower (Chapter 4) in the oviduct of molting hens compared with laying hens. Furthermore, expression of tight junction molecules *claudin-1, -3,* and *-5* mRNA and immunoreactive claudin-1 was reduced in the oviductal mucosa of molting hens (Chapter 5). These results suggest that the barrier function on the mucosal surface may be declined due to regression of mucin synthesis ability and tight junctional functions in the oviductal segments of molting hens.

Treatment of molting birds with EB caused oviductal growth with gain of weight and thickness of the surface epithelium. Mucin expression and density of AB-positive substances, namely mucopolysaccharides, were also increased in the surface

epithelium in both the vagina and uterus by EB treatment (Chapter 2). Expression of TLR4 and MyD88 were increased by EB-treated molting hens (Chapter 4). However, mucin expression after LPS treatment was significantly lower in vagina and uterus of EB-treated molting hens than in molting hens, suggesting that estrogen may suppress the response to LPS for mucin expression in the oviductal cells (Chapter 3). Injection of molting birds with estradiol upregulated the expression of tight junction molecules *claudin-1, -3*, and *-5* mRNA and claudin-1 protein in the mucosal epithelial cells (Chapter 5). Thus mucosal barrier system formed by mucin and tight junctions in the oviduct is likely to be enhanced by estrogen although response to LPS to induce mucin is downreguated by that steroid. Upregulation of expression of mucin and tight junction molecules by estrogen may be associated with the growth and differentiation of mucosal epithelium induced by that steroid.

Modulation of the innate immune response in the mucosa of hen oviduct may contribute in poultry production since the use of antibiotics has already been restricted because of potential development of antibiotics-resistant strains within food-poultry production (Lillehoj and Lee, 2012). With increasing consumer's concerns about drug-resistant microbes, new strategies for prevention of animal diseases that do not promote the development of antimicrobial resistance need to be developed. A large number of studies showed that many of these antibiotic alternatives, such as antimicrobial peptides, TLR ligands, probiotics, prebiotics, herbs, essential oils, and bacteriophages, can modulate host innate immunity (Lillehoj and Lee, 2012). Thus, innate immunity of mucosal barrier system of hen oviduct was also upregulated by bacterial LPS and estrogen as observed in this study.

In conclusion, this study showed that the hen oviduct forms a mucosal barrier system mediated by mucin and tight junction. This mucosal barrier system in the oviduct is expected to play important roles to protect the oviductal tissue and the eggs from infection by pathogenic microorganisms. The expressions of mucin and tight junction molecules were declined in molting hens with regression of oviduct and upregulated by estrogen. Thus, the mucosal barrier system formed by mucin and tight junction are probably weakened due to less circulating estrogen level. It was also established by this study that mucin expression was stimulated by LPS of Gram negative bacteria such as *Salmonella* organism through NFkB and AP-1 mediated manner in the oviduct.

Chapter 7

SUMMARY

Immune system in the oviduct is responsible to maintain the health of this organ to produce hygienic eggs. The mucosal barrier function plays essential roles in the defence to pathogens. Mucins, tight junctions of epithelium, and leukocyte activity form mucosal barrier to play roles to prevent infection in the mucosal tissues. Mucins may prevent adherence of pathogens to the mucosal surface. Tight junctions form the outer mechanical barrier to protect the mucosa from being invaded with infectious agents. The goal of this study was to determine the mechanism by which mucosal barrier mediated by mucins and tight junction is formed in the mucosal epithelium of the oviduct. Specifically, it was focused on the mechanism by which mucin synthesis for mucosal barrier is stimulated by oviductal growth, gonadal steroid and bacterial component, LPS, in the lower segment of oviduct, namely vagina, uterus or isthmus. Then, the existence of the epithelial barrier formed by tight junction was also examined.

1. Formation of mucosal surface barrier by mucin in the lower oviductal segments and its changes with egg-laying phase and gonadal steroid stimulation in hens.

Mucins produced by mucosal epithelial cells have the ability to form a physical barrier and act as adhesion decoys to invading agents. The aim of this study was to determine the effects of the egg-laying phase and estradiol on the mucin expression that forms a mucosal surface barrier in the lower oviductal segments in hens. White Leghorn laying and molting hens were used. Molting hens were given either sesame oil (control groups) or estradiol benzoate (EB groups) via i.m. injection (n = 5 per group). The lower segments of oviduct (vagina, uterus, and isthmus) of these birds were collected. Localization and gene expression of mucosal mucin were analyzed by quantitative RT-PCR and immunohistochemistry. Localization of mucin polysaccharide was performed by alcian blue (AB) staining. Sugar residues were localized by lectin (WGA or Jacalin) histochemistry. In the vagina, uterus and isthmus, mucin expression

was formed, and immunoreactive mucin5AC and AB-positive mucopolysaccharide were localized in the mucosal epithelium. Their expression and densities were reduced in molting hens compared with laying hens, and up-regulated by EB. Substances positively stained by WGA and Jacalin were identified on the surface of the mucosal epithelium in the lower oviductal segments in laying and molting hens. These results suggest that mucin synthesis in the lower segments of the oviduct is reduced due to decline of circulating estrogen level, although the existence of WGA- and Jacalin-positive sugars may be kept even in the molting phase.

2. Induction of mucin expression by lipopolysaccharide in the lower oviductal segments in hens

The aim of this study was to determine the effect of lipopolysaccharide (LPS), a component of Gram negative bacteria, on the mucin expression in the lower oviductal segments (vagina and uterus) of hens. The mucosal tissues of the vagina and uterus were collected from White Leghorn laying and molting hens, and molting hens with or without intramuscular injection with 1 mg estradiol-benzoate (EB) daily for 7 d. These tissues were cultured in TCM-199 culture medium with or without LPS (0, 10, 100 or 1000 ng/ml) for 1.5 or 3 h. Then, mucin expression was analyzed by quantitative RT-PCR. Cultured tissues were also processed for paraffin sections and stained with alcian blue (AB). Mucin expression in the cultured vagina and uterus tissues of laying and molting hens was up-regulated by LPS in a dose- and time-dependent manner. However, there was no significant response to LPS for induction of mucin in the tissues of EB-group hens. These results suggest that mucin expression responsible for mucosal barrier is stimulated by LPS in the vagina and uterus of both laying and molting hens. Estrogen may suppress the response to LPS for mucin induction.

3. Toll-like receptor signaling for the induction of mucin expression in response to lipopolysaccharide in hen vagina

TLRs are known to recognize microbial molecular patterns, generally by direct interaction with molecules on the pathogen surface. TLR-4 works to recognize lipopolysaccharide (LPS) of Gram-negative bacteria. The aim of this study was to

determine the intracellular signaling molecules for mucin induction by LPS, and the effect of molting and estrogen on their expression. Expression of TLR4, its adaptor molecules, and transcriptional factors in the vaginal mucosa of laying and molting hens treated with or without estradiol were examined by RT-PCR. Expression of mucin in the cultured mucosal tissue stimulated by LPS together with inhibitors of transcriptional factors was analyzed by quantitative RT-PCR. Expression of TLR4, its adaptor molecule, namely, myeloid differentiation factor 88 (MyD88) or toll-interleukin 1 receptor domain-containing adaptor-inducing IFN-β (TRIF), and transcriptional factors, namely, cFos, and cJun, were declined in molting hens compared with laying hens, and was upregulated by estradiol. In mucosal tissue of laying hens, mucin expression was upregulated by LPS, whereas it was suppressed by inhibitors of transcriptional factors, namely, ALLN (an inhibitor of IκB proteolysis), BAY-117085 (a NFκB inhibitor), U0126 (a mitogen-activated protein kinase [MAPK] inhibitor), and Transhinone IIA (an activated protein 1 [AP-1] inhibitor). These results suggest that MyD88-dependent pathway in the downstream of TLR4 and transcriptional factor of NFκB and AP-1 participate in the induction of mucin expression by LPS in the vaginal mucosa. Also, these signaling functions may be declined during molting due to the decline of circulating estrogen level.

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

Tight junction in the mucosal epithelium plays essential roles as a mucosal barrier to prevent the invasion of microbes into the mucosal tissues. The aim of this study was to determine the effects of egg-laying phase and gonadal steroid on the expression of tight junction molecule “claudins” in the lower oviductal segments in hens. White Leghorn laying and molting hens were used. A proportion of the molting hens were injected with sesame oil (control) or estradiol benzoate (EB). 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 *IFNγ* was analyzed by quantitative RT-PCR, and localization of claudin-1 was examined by immunohistochemistry. Permeability in the mucosal epithelium was examined by intrauterine injection of fluorescein isothiocyanate (FITC)-dextran. Expression of

claudin-1, -3, and *-5* genes and density of claudin-1 protein in the lower oviductal segments were significantly higher in laying hens than in molting hens, and their expression was upregulated by EB. Expression of *LITAF* and *IFNγ* genes was higher in molting hens than in laying hens. More FITC-dextran infiltrated into the intercellular space of the uterus mucosal epithelium in molting group hens than in laying and EB group hens. These results suggest that barrier functions of the mucosal epithelium at the lower oviductal segments may be disrupted due to reduction of claudin expression in molting hens.

5. Conclusion

This study has identified that the mucosal barrier system mediated by mucin and tight junction is formed in hen oviduct. This mucosal barrier system in the oviduct is expected to play important roles to protect the oviductal tissue from infection by pathogenic microorganisms. The expressions of mucin and tight junction molecules were declined in molting hens with regression of oviduct and upregulated by estrogen. Thus, the mucosal barrier system formed by mucin and tight junction are probably weakened due to less circulating estrogen level. It was also established by this study that mucin expression was stimulated by LPS of Gram negative bacteria such as *Salmonella* organism through NFkB and AP-1 mediated manner in the oviduct.

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REFERENCES

Abou El Azab MF, Fukushima Y, Horiuchi H, Matsuda H, and Furusawa S. Prolonged suppression of chick humoral immune response by antigen specific maternal antibody. *Journal of Veterinary Medical Science*, 71: 417-424. 2008.

Aitken RNC. The oviduct. In: Physiology and biochemistry of the domestic fowl. Vol. 3. DJ bell and BM Freeman, eds. Academic press, New York, pp. 1237-1289. 1971.

Aliprantis AO, Yang RB, Mark MR, Suggett S, Devaux B, Radolf JD, Klimpel GR, Godowski P, and Zychlinsky A. Cell activation and apoptosis by bacterial lipoproteins through Toll-like receptor-2. *Science*, 85: 736-739. 1999.

Angelow S, Ahlstrom R, and Yu AS. Biology of claudins. *American Journal of Physiology, Renal Physiology*, 295: 867-876. 2008.

Answers.com. http://www.answers.com/topic/poultry-production.

Bahr JM. The chicken ovary as a model of follicular development. *Seminars in Reproductive Endocrinology*, 9: 352-359. 1991.

Baker OJ, Camden JM, Redman RS, Jones JE, Seye CI, and Erb L. Proinflammatory cytokines tumor necrosis factor-α and interferon-ɣ alter tight junction structure and function in the rat parotid gland Par-C10 cell line. *American Journal of Physiology, Cell Physiology*, 295:1191-1201. 2008.

Bakst MR. Anatomical basis of sperm storage in the avian oviduct. *Scanning Microscopy*, 1:1256-1266. 1987.

Bara J, Forque-Lafitte ME, Maurin N, Flejou JF, and Zimber A. Abnormal expression of gastric mucin in human and rat aberrant crypt foci during colon carcinogenesis. *Tumour Biology*, 24:109-115. 2003.

Barton GM and Medzhitov R. Control of adaptive immune response by Toll-like receptors. *Current Opinion in Immunology*, 14: 380-383. 2002.

Berger RR and Sanders MM. Estrogen modulates HNF-3beta mRNA levels in the developing chick oviduct. *DNA and Cell Biology*, 19:103-112. 2000.

Berry M, Harris A, Lumb R, and Powell K. Commensal ocular bacteria degrade mucins. *The British Journal of Ophthalmology*, 86:1412-1416. 2002.

Blanchard AA, Watson PH, Shiu RP, Leygue E, Nistor A, and Wong P. Differential expression of claudin 1, 3, and 4 during normal mammary gland development in the mouse. *DNA and Cell Biology*, 25: 79-86. 2006.

Blanchard AA, Skliris GP, Watson PH, Murphy LC, Penner C, and Tomes L. Claudins 1, 3, and 4 protein expression in ER negative breast cancer correlates with markers of the basal phenotype. *Virchows Archiv*, 454:647-656. 2009.

Brownlie R, Zhu J, Allan B, Mutwiri GK, Babiuk LA, Potter A, and Griebel P. Chicken TLR21 acts as a functional homoloque to mammalian TLR9 in the recognition of CpG oligodeoxynucleotides. *Molecular Immunology*, 46: 3163-3170. 2009.

Bruewer M, Luegering M, Kucharzik T, Parkos CA, Madara JL, and Hopkins AM. Proinflammatory cytokines disrupt epithelial barrier function by apoptosis-independent mechanisms. *Journal of Immunology*, 171: 6164-6172. 2003.

Buckley M, Xin P, Washington S, Herb N, Erickson D, and Bhavanandan VP. Lectin histochemical examination of rabbit bladder glycoproteins and characterization of a mucin isolated from the bladder mucosa. *Archives of Biochemistry and Biophysics*, 375: 270-277. 2000.

Buisine MP, Devisme L, Savidge TC, Gespach C, Gosselin B, Porchet N, and Aubert JP. Mucin gene expression in human embryonic and fetal intestine. *Gut*, 43: 519-524. 1998.

Burek M, Arias-Loza PA, Roewer N, and Förster CY. Claudin-5 as a novel estrogen target in vascular endothelium. *Arteriosclerosis Thrombosis, and Vascular Biology*, 30: 298-304. 2010.

Capua I and Alexander DJ. Avian influenza: recent development. *Avian Pathology*, 33: 393-404. 2004.

Carlstedt I, Lindgren H, and Sheehan JK. The macromolecular structure of the human cervical-mucus glycoproteins. *The Biochemical Journal*, 213: 427-435. 1983.

Chappell L, Kaiser P, Barrow P, Jones MA, Johnston C, and Wigley P. The immunobiology of avian systemic *Salmonellosis*. *Veterinary Immunology and Immunopathology*, 128: 53-59. 2008.

Chiang HI, Berghman LR, and Zhou H. Inhibition of NF-kB 1 (NF-kBp50) by RNA interference in chicken macrophage HD11 cell line challenged with *Salmonella enteritidis. Genetics and Molecular Biology*, 32: 507-515. 2009.

Chousalkar KK and Roberts JR. Ultrastructural study of infectious bronchitis virus infection in infundibulum and magnum of commercial laying hens. *Veterinary Microbiology*, 122:223-236. 2007.

Cohen ML and Tauxe RV. Drug-resistant *Salmonella* in the United States: An epidemiologic perspective. *Science*, 234: 964-969. 1986.

Corfield AP, Myerscough N, Longman R, Sylvester P, Arul S, and Pignatelli M. Mucins and mucosal protection in the gastrointestinal tract: new prospects for mucins in the pathology of gastrointestinal disease. *Gut*, 47: 589-594. 2000.

Das SC, Isobe N, and Yoshimura Y. Changes in the expression of interleukin-1β and lipopolysaccharide-induced TNF factor in the oviduct of laying hens in response to artificial insemination. *Reproduction*, 137: 527-536. 2009.

De Buck J, Pasmans F, Van Immerseel F, Haesebrouck F, and Ducatelle R. Tubular glands of the isthmus are the predominant colonization site of *Salmonella enteritidis* in the upper oviduct of laying hens. *Poultry Science*, 83:352-358. 2004a.

De Buck J, Van Immerseel F, Haesebrouck F and Ducatelle R. Colonization of the chcken reproductive tract and egg contamination by *Salmonella*. *Journal of Applied Microbiology*, 97: 233-245. 2004b.

Desseyn JL, Aubert JP, Porchet N, and Laine A. Evolution of the large secreted-gel forming mucins. *Molecular Biology and Evolution*, 17:1175-1184. 2000.

de Zoete MR, Keestra AM, Roszczenko P, and van Putten JP. Activation of human and chicken Toll-like receptor by *Campylobacter spp*. *Infection and Immunity*, 78:1229-1238. 2010.

de Zoete MR, Bouman LI, Keestra AM, and van Putten JP. Cleavage and activation of Toll-like receptor by microbial proteases. *Proceeding of the National Academy of Sciences of the United States of America*. 108: 4968-4973. 2011

Dougherty DC and Sanders MM. Estrogen action: revitalization of the chick oviduct model. *Trends in Endocrinology Metabolism*, 16: 414-419. 2005.

Domermuth CH, Cross WB, and Bose RT. Mycoplasmal salpingitis of chickens and turkeys. *Avian Diseases*, 11:393-398. 1967.

Dunkley CS, McReynolds JL, Dunkley KD, Njongmeta LN, Berghman LR, and Kubena LF. Molting in *Salmonella enteritidis*-challenged laying hens fed alfalfa crumbles. IV. immune and stress protein response. *Poultry Science*, 86:2502–2508. 2007.

Dunzendorfer S, Lee HK, Soldau K, and Tobias PS. TLR4 is the signaling but not the lipolysaccharide uptake receptor. *Journal of Immunology*, 173:1166-1170. 2004.

Fallis LC, Stein KK, Lynn JW, and Misamore MJ. Identification and role of carbohydrates on the surface of gametes in the zebra mussel, *Dreissena polymorpha. The Biological Bulletin*, 218: 61-74. 2010.

Forster C. Tight junctions and the modulation of barrier function in disease. *Histochemistry and Cell Biology*, 130: 55-70. 2008.

Fujii S and Tamura T. Location of sperms in oviduct of the domestic fowl with special reference to storage of sperms in the vaginal gland. *Journal of Faculty of Fisheries and Animal Husbandry, Hiroshima University*. 5:145-163. 1963.

Fukui A, Inoue N, Matsumoto M, Nomura M, Yamada K, Matsuda Y, Toyoshima K, and Seya T. Molecular cloning and functional characterization of chicken Toll-like receptors. A single chicken toll covers multiple molecular patterns. *The Journal of Biological Chemistry*, 276: 47143-47149. 2001.

Ganz T. The role of antimicrobial peptides in innate immunity. *Integrative and Comparative Biology*, 43: 300-304. 2003.

Gendler SJ and Spicer AP. Epithelial mucin genes. *Annual Review of Physiology*, 57: 607-634. 1995.

Ghisletti S, Meda C, Maggi A, and Vegeto E. 17beta-estradiol inhibits inflammatory gene expression by controlling NF-kappaB intracellular localization. *Molecular and Cellular Biology*, 25: 2957-2968. 2005.

Gilbert AB. Egg albumin and its formation. In physiology and biochemistry of domestic fowl. (Bell DJ and Freeman BM eds). pp. 1291-1330. Academic press, London. 1971.

Gipson IK. Mucin genes expressed by human female reproductive tract epithelia. *Biology of Reproduction*, 56: 999-1011. 1997.

Golden NJ, Marks HH, Coleman ME, Schroeder CM, Bauer Jr NE, and Schlosser WD. Review of induced molting by feed removal and contamination of eggs with *Salmonella enterica* serovar *enteritidis*. *Veterinary Microbiology*, 131: 215-228. 2008.

Gollub EG, Waksman H, Goswami S, and Marom Z. Mucin genes are regulated by estrogen and dexamethasone. *Biochemical and Biophysical Research Communication*, 217:1006-1014. 1995.

González-Mariscal L, Betanzos A, Nava P, Jaramillo BE. Tight junction proteins. *Progress in Biophysics and Molecular Biology*, 81:1-44. 2003.

Gorodeski GI. Estrogen decrease in tight junctional resistance involves matrix-metalloproteinase-7-mediated remodeling of occludin. *Endocrinology*, 148: 218-231. 2007.

Goto Y and Kiyono H. Epithelial barrier: an interface for the cross-communication between gut flora and immune system. *Immunological Reviews*, 245:147-163. 2012.

Haworth KE, El-Hanfy A, Prayag S, Healy C, Dietrich S, and Sharpe P. Expression of claudin-3 during chick development. *Gene Expression Patterns*, 6: 40-44. 2005.

Heil F, Hemmi H, and Hochrein H. Species-specific recognition of single-stranded RNA via Toll-like receptor 7 and 8. *Science*, 303:1526-1529. 2003.

Hemmi H, Kaisho T, Takeuchi O, Sato S, Sanjo H, Hoshino K, Horiuchi T, Tomizawa H, Takeda K, and Akira S. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nature Immunology*, 3:196-200. 2002.

Higgs R, Cormican P, Cahalane S, Allan B, Lloyd AT, Meade K, James T, Lynn DJ, Babiuk LA, and Farrelly C. Induction of a novel chicken Toll-like receptor following *Salmonella enterica* serovar *typhimurium* infection. *Infection and Immunity*, 74:1692-1698. 2006.

Hodges RD. The histology of the fowl. 1st Edition. Academic Press, London, pp. 348-358. 1974

Hofstad MS, Calnek BW, Helmboldt CF, Reid WM, and Yoder Jr HW. Disease of Poultry. 7th Edition. Iowa State University Press, Ames, Iowa, pp. 619-630. 1978.

Holt PS. Molting and *Salmonella enterica* serovar *enteritidis* infection: the problem and some solutions. *Poultry Science*, 82:1008-1010. 2003.

Hopkins PA and Sriskandan S. Mammalian Toll-like receptors: to immunity and beyond. *Clinical and Experimental Immunology*, 140: 395-407. 2005.

Hornef MW, Normark BH, and Vandewalle A. Intracellular recognition of lipopolysaccharide by Toll-like receptor 4 in intestinal epithelial cells. *The Journal of Experimental Medicine*, 198: 1225-1235. 2003.

Hornung V, Rothenfusser S, and Britsch. Quantitative expression of Toll-like receptors 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *Journal Immunology*, 168: 4531-4537. 2002.

Hossain MS, Chowdhury EH, Islam MM, Haider MG, and Hossain MM. Avian Salmonella infection: isolation and identification of organisms and histopathological study. *Bangladesh Journal of Veterinary Medicine*, 4: 7-12. 2006.

Howard ZR, O'bryan CA, Crandall PG, and Ricke SC. *Salmonella enteritidis* in shell eggs: Current issues and prospects for control. *Food Research International*, 45: 755-764. 2012.

Iqbal M, Philbin VJ, Withanage GS, Wigley P, Beal PRK, Goodchild MJ, Barrow P, McConnell I, Maskell DJ, Young J, Bumstead N, Boyd Y, and Smith AL. Identification and functional characterization of chicken Toll-like receptor 5 reveals a fundamental role in the biology of infection with *Salmonella enterica* serovar *typhimurium*. *Infection and Immunity*, 73: 2344-2350. 2005.

Itoh M and Bissell MJ. The organization of tight junctions in epithelia: implications for mammary gland biology and breast tumorigenesis. *Journal of Mammary Gland Biology and Neoplasia*, 8: 449-462. 2003.

Johnson AL. Reproduction in the female. In: Sturkie's Avian Physiology (Whittow GC ed.) 5th ed. pp.569-596. Academic Press. San Diego. 2000.

Johnston HS, Aitken RNC, and Wyburn GM. The fine structure of the uterus of the domestic fowl. *Journal of Anatomy*, 97: 333-334. 1963.

Jones MA, Wigley P, Page KL, Hulme SD, and Barrow PA. Salmonella enterica serovar gallinarum requires the salmonella pathogenicity island 2 type III secretion system but not the Salmonella pathogenicity island 1 type III secretion system for virulence in chickens. *Infection and Immunity*, 69: 5471-5476. 2001.

Jung JG, Lim W, Park TS, Kim JN, Han BK, Song G, and Han JY. Structural and histological characterization of oviductal magnum and lectin-binding patterns in *Gallus domesticus*. *Reproductive Biology and Endocrinology*, 9: 1-11. 2011.

Kabashima K, Saji T, Murata T, Nagamachi M, Matsuoka T, and Segi E. The prostaglandin receptor EP4 suppresses colitis, mucosal damage and CD4 cell activation in the gut. *The Journal of Clinical Investigation*, 109: 883-893. 2002.

Kabir S. Jacalin: a jackfruit (*Artocarpus heterophyllus*) seed-derived lectin of versatile applications in immunobiological research. *Journal of Immunological Methods*, 212: 193-211. 1998.

Kaiser MG and Lamont SJ. Genetic line differences in survival and pathogen load in young layer chicks after *Salmonella* serovar *enteritidis* exposure. *Poultry Science*, 80: 1105-1108. 2001.

Kami K and Yasuda K. Immunoelectron microscopical demonstration of endogenous avidin in secretory granules of the hen oviduct mucosa: A preliminary study. *The histochemical Journal*, 16: 835-842. 1984.

Kawai T and Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature Immunology*, 11: 373-384. 2010.

Keestra AM and van Putten JP. Unique properties of chicken TLR4/MD-2 complex: selective lipopolysaccharide activation of the MyD88-dependent pathway. *Journal of Immunology*, 181: 4354-4362. 2008.

Keestra AM, de Zoete MR, Bouwman LI, Vaezirad MM, and van Putten JP. Unique features of chicken Toll-like receptors. *Developmental and Comparative Immunology*, 41: 316-323. 2013.

Keller LH, Benson CE, Krotec K, and Eckroade RJ. *Salmonella enteritidis* colonization of the reproductive tract and forming and freshly laid eggs of chickens. *Infection and Immunity*, 63: 2443-2449. 1995.

Keymer IF. Disorders of the avian female reproductive system. *Avian Pathology*, 9: 405-419. 1980.

Kogut MH, Iqbal M, He H, Philbin V, Kaiser P, and Smith A. Expression and function of Toll-like receptors in chicken heterophils. *Developmental Comparative Immunology*, 29: 791-807. 2005.

Kogut MH, Chiang HI, Swaggerty CL, Pevzner IY, and Zhou H. Gene expression analysis of toll-like receptor pathways in heterophils from genetic chicken lines that differ in their susceptibility to *Salmonella enteritidis*. *Frontiers in Genetics*, 3: 1-10. 2012.

Krause G, Winkler L, Mueller SL, Haseloff RF, Piontek J, and Blasig IE. Structure and function of claudins. *Biochimica et Biophysica Acta*, 1778: 631-645. 2008.

Kurt-Jones EA, Chan M, Zhou S, Wang J, Reed G, Bronson R, Arnold MM, Knipe DM, and Finberg RW. Herpes simplex virus 1 interaction with Toll-like receptor 2 contributes to lethal encephalitis. *Proceeding of the National Academy of Sciences of the United States of America*, 101: 1315-1320. 2004.

Lemaitre B, Nicolas E, Michaut L, Reichhart JM, and Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in drosophila adults. *Cell*, 86: 973-983. 1996.

Levy AM, Izumiya Y, Brunovskis P, Xia L, Parcells MS, Reddy SM, Lee L, Chen HW, and Kung HJ. Characterization of the chromosomal binding sites and dimerization partners of the viral oncoprotein Meq in Marek's disease virus-transformed T cells. *Journal of Virology*, 77: 12841-12851. 2003.

Li Q, Zhang Q, Wang M, Zhao S, Ma J, and Luo N. Interferon-γ and tumor necrosis factor-alpha disrupt epithelial barrier function by altering lipid composition in membrane microdomains of tight junction. *Journal of Clinical Immunology*, 126: 67-80. 2008.

Lillehoj HS and Lee KW. Immune modulation of innate immunity as alternative-to-antibiotics strategies to mitigate the use of drugs in poultry production. *Poultry Science*, 91: 1286-1291. 2012.

Lillehoj HS and Okumura M. Host immunity and vaccine development to coccidian and Salmonella infections in chickens. *Journal of Poultry Science*, 40: 151-193. 2003.

Linden SK, Florin THJ, and McGuckin MA. Mucin dynamics in intestinal bacterial infection. *PLoS One* 3: 1-14. 2008a.

Linden SK, Sutton P, Karlsson NG, Korolik V, and McGuckin MA. Mucin in the mucosal barrier to infection. *Mucosal Immunology*, 1: 183-197. 2008b.

Lindgren NO. On the aetiology of salpingitis and salpingo-peritonitis of the domestic fowl. A statistical and experimental investigation. *Stockholm Akademisk Avhandling*. 1964.

Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods,* 25: 402-408. 2001.

Macia L, Thornburn AN, Binge LC, Marino E, Rogers KE, Maslowski KM, Vieira AT, Kranich J, and Mackay CR. Microbial influences on epithelial integrity and immune function as a basis for inflammatory diseases. *Immunological Reviews*, 245: 164-176. 2012.

Makita T, Kiwaki S, and Sandborn EB. Secretion granules in the ciliated cells of the avian oviduct. *Archivum Histologicum Japonicum*, 35: 209-224. 1973.

Martin del las Mulas J, Fernandes A, Sierra MA, Poveda JB and Carranza J. Immunohistochemical demonstration of Mycoplasma gallinarum and Mycoplasma gallinaceum in naturally infected hen oviducts. *Research in Veterinary Science*, 49: 339-345. 1990.

Mazzon E and Cuzzocrea S. Role of TNF-α in ileum tight junction alteration in mouse model of restraint stress. *American Journal of Physiology Gastrointestinal and Liver Physiology*, 294: 1268-1280. 2008.

Medzhitov R. Toll-like receptors and innate immunity. *Nature Reviews, Immunology*, 1: 135-145. 2001.

Moore RW and Holt PS. The effect of feed deprivation on tissue invasion by *Salmonella enteritidis*. *Poultry Science*, 85: 1333-1337. 2006.

Munro SS and Kosin TL. Dramatic response of the chick oviduct to estrogen. *Poultry Science*, 22: 330-331. 1943.

Murphy K, Travers P, and Walport M. Janeway's immunobiology. 7th ed. *Garland Science, Taylor and Francis Group*, New York and London. 2007

NCBI. http://www.ncbi.nlm.nih.gov/nuccore?term=gallus%20gallus%20claudin. Accession date July 11, 2012.

Nerren JR, He H, Genovese K, and Kogut MH. Expression of the avian-specific Toll-like receptor 15 in chicken heterophils is mediated by Gram-negative and Gram-positive bacteria, but not TLR agonists. *Veterinary Immunology and Immunopathology*. 136: 151-156. 2010.

Nie YC, Wu H, Li PB, Luo YL, Zhang CC, Shen JG, and Su WW. Characteristic comparison of three rat models induced by cigarette smoke or combined with LPS: to establish a suitable model for study of airway mucus hypersecretion in chronic obstructive pulmonary disease. *Pulmonary Pharmacology and Therapeutics*, 25: 349-356. 2012.

Nii T, Sonoda Y, Isobe N, and Yoshimura Y. Effects of lipopolysaccharide on the expression of proinflammatory cytokines and chemokines and the subsequent recruitment of immunocompetent cells in the oviduct of laying and molting hens. *Poultry Science*, 90: 2332-2341. 2011.

Nii T, Isobe N, and Yoshimura Y. Effects of repeated lipopolysaccharide stimulation on the development of antigen-presenting cells and T cells pool in hen vagina. *The Journal Poultry Science*, 50: 83-89. 2013.

Oka T and Schimke RT. Interaction of estrogen and progesterone in chick oviduct development. I. antagonistic effect of progesterone on estrogen-induced proliferation and differentiation of tubular gland cells. *The Journal of Cell Biology*, 41: 816-831. 1969.

O'Neill LA and Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signaling. *Nature Reviews Immunology*, 5: 353-364. 2007.

Ozden O, Black BL, Ashwell CM, Tipsmark CK, Borski RJ, and Grubb BJ. Developmental profile of claudin-3, -5 and -16 proteins in the epithelium of chick intestine. *Anatomical Record*, 293: 1175-1183. 2010.

Ozinsky A, Underhill DM, and Fontenot JD. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. *Proceedings of the National Academy of Sciences of the United States of America,* 97: 13766-13771. 2000.

Ozoe A, Isobe N, and Yoshimura Y. Expression of Toll-like receptors (TLRs) and TLR4 response to lipopolysaccharide in hen oviduct. *Veterinary Immunology and Immunopathology*, 127: 259-268. 2009.

Panda B. Structure and problems of the poultry industry in Southern Asia. *World's Poultry Science Journal*, 45: 66-71. 1989.

Park CJ, Lee JE, Oh YS, Shim S, Nah WH, and Choi KJ. Expression of claudin-1 and -11 in immature and mature pheasant (*Phasianus colchicus*) testes. *Theriogenology,* 75: 445-458. 2011.

Paszkiewicz-Gadek A, Porowska H, Anchim T, Wolczynski S, and Gindzienski A. Biosynthesis of MUC1 mucin in human endometrial adenocarcinoma is modulated by estradiol and tamoxifen. *Gynecological Endocrinology*, 17: 37-44. 2003.

Perez-Vilar J. Mucin granule intraluminal organization. *American Journal of Respiratory Cell and Molecular Biolology*, 36: 183-190. 2007.

Philbin VJ, Iqbal M, Boyd Y, Goodchild MJ, Beal RK, Bumstead N, John Y, and Smith AL. Identification and characterization of a functional, alternatively spliced Toll-like receptor 7 (TLR7) and genomic disruption of TLR8 in chickens. *Immunology*, 114: 385-391. 2005.

Porchet N, Pigny P, Buisine MP, Bebailleul V, Degand P, Laine A, and Aubert JP. Human mucin genes: genomic organization and expression of MUC4, MUC5AC, and MUC5B. *Biochemical Society Transactions*, 23: 800-805. 1995.

Promkuntod N, Antarasena C, Prommuang P and Prommuang P. Isolation of avian influenza virus A subtype H5N1 from internal contents (albumen and allantoic fluid) of Japanese quail (*Coturnix coturnix japonica*) eggs and oviduct during a natural outbreak. *Annal of the New York Academy of Sciences*, 1081: 171-173. 2006.

Ricke SC, Dunkley CS, and Durant JA. A review on development of novel strategies for controlling *Salmonella enteritidis* colonization in laying hens: fiber-based molt diets. *Poultry Science*, 92: 502-525. 2013.

Sato K, Takahashi K, Tohno M, Miura Y, Kamada T, and Ikegami S. Immunomodulation in gut-associated lymphoid tissue of neonatal chicks by immunobiotic diet. *Poutry Science*, 88: 2532-2538. 2009.

Shen H, Yoshida H, Yan F, Li W, Xu F, Huang H, Jono H, and Li JD. Synergistic induction of MUC5AC mucin by nontypeable *Haemophilus influenzae* and

Streptococcus pneumoniae. *Biochemical and Biophysical Research Communications*, 365: 795-800. 2008.

Simard A, Di Pietro E, Young CR, Plaza S, and Ryan AK. Alterations in heart looping induced by overexpression of the tight junction protein claudin-1 are dependent on its C-terminal cytoplasmic tail. *Mechanisms and Development*, 123: 210-227. 2006.

Shambaugh GE, Koehler RR, and Radosevich RR. Nutrition and fetal brain maturation II. impact of maternal starvation on changing levels of acetylcholinesterase and enolase in vitro. *Experimental Cell Research*, 175: 344-353. 1988.

Sharma DN and Singh CM. Studies on pathology of female genital tract of poultry with special reference to egg peritonitis incidence, patho-anatomy and experimental study. *Indian Journal of Veterinary Science and Animal Husbandry*, 38: 737-746. 1968.

Sharma JM. Overview of the avian immune system. *Veterinary immunology and Immunopathology*, 30: 13-17. 1991.

Shivaprasad HL. Fowl typhoid and pullorum disease. *Revue Scientifique et Technique*, 19: 405-424. 2000.

Simkiss K. The structure and formation of the shell and shell membranes. In: Egg quality: A study of the hen's egg (Carter TC, ed.). Oliver and Boyd, Eidenburgh. 1968.

Solomon SE. Study on the isthmus region of the domestic fowl. *British Poultry Science*, 16: 255-258. 1975.

Smirnova MG, Guo L, Birchall JP, and Pearson JP. LPS up-regulates mucin and cytokine mRNA expression and stimulates mucin and cytokine secretion in goblet cells. *Cellular Immunology*, 221: 42-49. 2003.

Smirnov A, Sklan D, and Uni Z. Mucin dynamics in the chick small intestine are altered by starvation. *The Journal of Nutrition*, 134: 736-742. 2004.

Smirnov A, Perez R, Amit-Romach E, Sklan D, and Uni Z. Mucin dynamics and microbial populations in chicken small intestine are changed by dietary

probiotic and antibiotic growth promoter supplementation. *The Journal of Nutrition*, 135: 187-192. 2005.

Smirnov A, Tako E, Ferket PR, and Uni Z. Mucin gene expression and mucin content in the chicken intestinal goblet cells are affected by in ovo feeding of carbohydrates. *Poultry Science*, 85: 669-673. 2006.

Song KS, Kim HJ, Kim K, Lee JG, and Yoon JH. Regulator of G-protein signaling 4 suppresses LPS-induced MUC5AC overproduction in the airway. *American Journal of Respiratory Cell and Molecular Biology*, 41: 40-49. 2009.

Stemberger BH, Muller WJ, and Leach RM. Microscopic study of the initial stages of the shell calcification. *Poultry Science*, 56: 537-543. 1977.

St Paul M, Brisbin JT, Abdul-Careem MF, and Sharif S. Immunostimulatory properties of Toll-like receptor ligands in chickens. *Veterinary Immunology and Immunopathology*, 152: 191-199. 2013.

Subedi K, Isobe N, Nishibori M, and Yoshimura Y. Changes in the expression of Toll-like receptor mRNA during follicular growth and in response to lipopolysaccharide in ovarian follicles of laying hens. *Journal of Reproduction and Development,* 53: 1227-1235. 2007.

Sundaresan NR, Anish D, Sastry KV, Saxena VK, Mohan J, and Saxena M. Differential expression of lipopolysaccharide-induced TNFα factor (LITAF) in reproductive tissues during induced molting of white leghorn hens. *Animal Reproduction Science*, 102: 335-342. 2007.

Sundaresan NR, Anish D, Sastry KV, Saxena VK, Nagarajan K, and Leo MD. High doses of dietary zinc induce cytokines, chemokines, and apoptosis in reproductive tissues during regression. *Cell and Tissue Research*, 332: 543-554. 2008.

Takata T, Liang J, Nakano H, and Yoshimura Y. Invasion of *Salmonella enteritidis* in the tissues of reproductive organs in laying Japanese quail: an immunocytochemical study. *Poultry Sciece*, 82: 1170-1173. 2003.

Takeda K and Akira S. Toll-like receptors. *Annual Review of Immunology*, 21: 335-376. 2003.

Takeuchi O, Kawai T, Muhlradt PF, Morr M, Radolf JD, Zychlinsky A, Takeda K, and Akira S. Discrimination of bacterial lipoproteins by Toll-like receptor 6. *International Immunology*, 13: 933-940. 2001.

Takeuchi O, Sato S, Horiuchi T, Hashino K, Takeda K, Dong Z, Modlin RL and Akira S. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *Journal of Immunology*, 169: 10-14. 2002.

Tatsuzuki A, Ezaki T, Makino Y, Matsuda Y, and Ohta H. Characterization of the sugar chain expression of normal term human placental villi using lectin histochemistry combined with immunohistochemistry. *Archives of Histology and Cytology*, 72: 35-49. 2009.

Tizard IR. Immunity in the fetus and newborn. In: veterinary immunobiology- an introduction (Tizard, IR ed.) pp.221-233. Philadelphia. 2004.

Tsukita S, Furuse M, and Itoh M. Multifunctional strands in tight junctions. *Molecular Cel Biology, Nature Reviews*, 2: 285-293. 2001.

Tuohimaa P, Joensuu T, Isola J, Keinanen R, Kunnas T, Niemela A, Pekk A, Wallen M, Ylikomi T, and Kulomaa. Developmental of progestin-specific response in the chick oviduct. *The International Journal of Developmental Biology*, 33: 125-134. 1989.

Turksen K and Troy TC. Barriers built on claudins. *Journal of Cell Science*, 117: 2435-2447. 2004.

Ulevitch RJ. Therapeutics targeting the innate immune system. *Nature Reviews, Immunology*, 4: 512-520. 2004.

Utsumi T and Yoshimura Y. Applicability of lectin histochemistry in a test system with in ovo treatment for detecting androgenic and antiandrogenic effects of chemicals in japanese quail (*Coturnix japonica*). *Poultry Science*, 90: 168-174. 2011.

Van Italie CM and Anderson JM. Claudins and epithelial paracellular transport. *Annual Reviews Physiology*, 68: 403-429. 2006.

Vanmontfort D, Rombauts L, Decuypere, and Verhoeven G. Source of immunoreactive inhibin in the chicken ovary. *Biology of Reproduction*. 47: 977-983. 1992.

Van Poucke SGM, Nicholls JM, Nauwynck HJ, and Van Reeth KV. Replication of avian, human and swine influenza viruses in porcine respiratory explants and association with sialic acid distribution. *Virology Journal*, 7: 38:1-14. 2010.

Wang Y, Shen Y, Li K, Zhang P, Wang G, Gao L, and Bai C. Role of matrix metalloproteinase-9 in lipopolysaccharide-induced mucin production in human airway epithelial cells. *Archives of Biochemistry and Biophysics*, 486: 111-118. 2009.

Wang W, Xu X, Zheng M, and Wan I. Lipopolysaccharides induces MUC5AC overproduction in human nasal epithelium. *European Archives of Otorhinolaryngology*, 270: 541-547. 2013.

Wigley P, Berchieri JR, Page KL, Smith AL, and Barrow PA. *Salmonella enterica* serovar *pullorum* persists in splenic macrophages and in the reproductive tract during persistent disease-free carriage in chickens. *Infection and Immunity*, 69: 7873-7879. 2001.

Will C, Fromm M, and Müller D. Claudin tight junction proteins: novel aspects in paracellular transport. *Peritoneal Dialysis International*, 28: 577-584. 2008.

Wyburn GM, Johnston HS, Draper MH, and Davidson MF. The ultrastructure of the shell-forming region of the oviduct and the development of the shell of *Gallus domesticus*. *Experimental Physiology*, 58: 143-151. 1973.

Yamamoto T, Ozawa H, and Nagai H. Histochemical studies of Ca-ATPase, succinate and NAD+ -dependent isocitrate dehydrogenases in the shell gland of laying Japanese quails with special reference to calcium-transporting cells. *Histochemistry*, 83: 221-226. 1985.

Yang BC, Yang ZH, Pan XJ, Xiao FJ, Liu XY, Zhu MX, and Xie JP. Crotonaldehyde-exposed macrophages induce IL-8 release from airway epithelial cells through NFκB and AP-1 pathways. *Toxicology Letters*, 219: 26-34. 2013.

Yarovinsky F, Zhang D, Andersen JF, Bannenberg GL, Serhan CN, Hayden MS, Hieny S, Sutterwala FS, Flavel RA, Ghosh S, and Sher A. TLR11 activation of

dendritic cells by protozoan profiling-like protein. *Science*, 308: 1626-1629. 2005.

Yilmaz A, Shen S, Adelson DL, Xavier S and Zhu JJ. Identification and sequence analysis of chicken Toll-like receptors. *Immunogenetics*, 56: 743-753. 2005.

Yoshimura Y, Okamoto T, and Tamura T. Localization of MHC class II, lymphocytes and imunoglobulins in the oviduct of laying and molting hens. *British Poultry Science,* 38: 590-596. 1997.

Zeng R, Li X, and Gorodeski GI. Estrogen abrogates transcervical tight junctional resistance by acceleration of occludin modulation. *The Journal of Clinical Endocrinology and Metabolism*, 89: 5145-5155. 2004.