ABSTRACT

OZDEN, OZKAN. Expression of Claudin Tight Junction Proteins in Response to Varying Environmental and Physiological Conditions. (Under the directions of Dr. Brenda Grubb and Dr. Betty Black.)

In this study, the localization and possible functions of claudin tight junction proteins in bird and fish gastrointestinal tract were investigated. Claudin expression was detected in the chick intestine during the last week of embryonic development using microarrays and quantitative real time PCR analyses. Expression profiles differed among the claudins suggesting that they might play different roles in the establishment of intestinal tight junctions during embryonic development. Glucocorticoids are essential for the maturation of numerous tissues during embryonic development and are known to increase the integrity of tight junctions. This study reveals that treatment of 18-day old embryonic intestine with the synthetic glucocorticoid dexamethasone elevates mRNA levels of two claudins within 12 hours.

Expression profiles of claudins were also analyzed in the gastrointestinal tract of Mozambique Tilapia. Immunohistochemistry and real-time PCR analyses revealed that various claudins were expressed in the tilapia gut in a tissue specific manner. Salinity transfer experiments indicated that salinity differentially regulates the expression of certain claudins suggesting a role for these proteins in osmoregulation.

Expression of Claudin Tight Junction Proteins in Response to Varying Environmental and Physiological Conditions

by
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BIOGRAPGY

Ozkan Ozden was born in 1978 in Ankara, Turkey. He started his undergraduate education in the department of Biology at Ankara University, Turkey in 1996. He graduated in 2000 and attended the Master of Science degree program in Zoology Department at North Carolina State University. He studied with Dr. Betty L. Black and Dr. Brenda J. Grubb on tight junction proteins in the chick intestine. After completing his master's program in 2004, he continued his Ph.D. education in the same program at North Carolina State University. He completed his PhD degree in December, 2008. He is planning to pursue his carrier at National Health Institute as a postdoctoral fellow.

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GENERAL INTRODUCTION

Tight Junctions and Claudins:

There are two pathways for the transport of solutes across the epithelial layers in many organs; transport through the epithelial cell membranes, called transcellular transport, and transport through the spaces between the cells called paracellular transport (Anderson, 2001; Tsukita et al., 2001). Tight junctions are located near the apical side of epithelial cells within the lateral plasma membranes and are responsible for the regulation of paracellular transport. In transmission electron micrographs, tight junctions appear as a series of close points between adjacent cell membranes, so-called kissing points (Farquar and Palade, 1963). Tight junctions are formed by three different types transmembrane proteins: those of the claudin protein family, occludin, and junctional adhesion molecules. Additionally, Zona Occludens (ZO-1, ZO-2, and ZO-3) are cytoplasmic adapter proteins that connect tight junctional transmembrane proteins to the cytoskeleton. The claudin protein family is believed to be one of the main structural and functional components of tight junctions, and includes more than 20 members whose amino acid sequences are evolutionary well conserved among vertebrates. Claudin proteins have 4 transmembrane domains; their short amino (2-6 amino acid residues) and carboxyl termini are located in the cytoplasm. They have two extracellular loops. The first extracellular loop is longer and more hydrophobic than the second one, and this includes highly conserved residues (W-GLW-C-C) (Van Itallie and Anderson, 2006; Gonzalez-Mariscal et. al., 2003). The first extracellular loop of claudins forms bridges between the cells. Claudins are one of the components that determine the size and charge specificity of pores in the paracellular spaces, but how they

polymerize at the tight junctional areas is not yet known (Krause et al., 2008; Van Itallie et. al., 2008; Gonzalez-Mariscal et. al., 2003; Colegio et al., 2002).

The main role of tight junctions. Called the barrier function, is to seal the paracellular spaces and prevent free diffusion of toxins and pathogens into the extracellular fluid (Powell, 1981). The tight junctional barrier is not absolute, and certain claudins form charge and size specific pore-like structures in the paracellular spaces and regulate the passage of molecules (Colegio et al., 2002). Secondly, tight junctions prevent lateral movement of membrane proteins and membrane lipids, and they separate the apical and the basolateral sides of epithelial layers. This polarization function of tight junctions is called the fence function (Tsukita et al., 2001). In addition, some claudins might contribute to adhesion between the cells, and some claudins could influence the activity of important signaling molecules (Tsukita and Furuse, 2000; Dhawan et al., 2005). Tight junctions are dynamic structures (Schneeberger and Lynch, 1992) and can change their expression and organization in response to different physiological conditions, such as proliferation, apoptosis, and metabolism (Takala et al., 2007; Bojarski et al., 2004; Kapus and Szaszi, 2006).

The majority of studies on claudins have been performed in mature mammalian tissues or cell cultures. In this study, subcellular localizations of claudin-like proteins in both fish and embryonic chicken were investigated because very few studies are currently present showing the localization of these evolutionary conserved proteins in the gastrointestinal epithelium of these organisms. The potential roles of certain claudins for osmoregulation in fish gastrointestinal tract epithelium were explored. Additionally, the

expression and regulation of various claudins in chick intestine during embryonic development was investigated. The contribution of claudins to the integrity of tight junctions, and ultimately to the overall permeability of the gastrointestinal epithelial layers, provides important information for understanding osmoregulation in fish as well as cell differentiation and functional development of the intestine in birds. This research should be of interest to a wide range of biologists in the fields of cell biology, developmental biology, and gastrointestinal physiology.

REFERENCES

Anderson JM. 2001. Molecular structure of tight junctions and their role in epithelial transport. News Physiol Sci. 16:126-30.

Bojarski C, Weiske J, Schöneberg T, Schröder W, Mankertz J, Schulzke JD, Florian P, Fromm M, Tauber R, Huber O. 2004. The specific fates of tight junction proteins in apoptotic epithelial cells. J Cell Sci. 117(Pt 10):2097-107.

Colegio OR, Van Itallie CM, McCrea HJ, Rahner C, Anderson JM. 2002. Claudins create charge-selective channels in the paracellular pathway between epithelial cells. Am J Physiol Cell Physiol. 283(1):C142-147.

Dhawan P, Singh AB, Deane NG, No Y, Shiou SR, Schmidt C, Neff J, Washington MK, Beauchamp RD. 2005. Claudin-1 regulates cellular transformation and metastatic behavior in colon cancer. J Clin Invest. 115(7):1765-1776.

Farquhar MG, Palade GE. 1963. Junctional complexes in various epithelia. J Cell Biol., 17:375-412.

Gonzalez-Mariscal, L., Betanzos, A., Nava, P. and Jaramillo, BE. 2003. Tight junction proteins. Prog. Biophys. Mol. Biol. 81, 1-44.

Kapus A, Szászi K. 2006. Coupling between apical and paracellular transport processes. Biochem Cell Biol. 84(6):870-880.

Krause G, Winkler L, Mueller SL, Haseloff RF, Piontek J, Blasig IE. 2008. Structure and function of claudins. Biochim Biophys Acta. 1778(3):631-645.

Powell DW. 1981. Barrier function of epithelia. Am J Physiol., 241(4):G275-88.

Schneeberger EE, Lynch RD. 1992. Structure, function, and regulation of cellular tight junctions. Am J Physiol., 262(6 Pt 1):L647-61.

Takala H, Saarnio J, Wiik H, Soini Y. 2007. Claudins 1, 3, 4, 5 and 7 in esophageal cancer: loss of claudin 3 and 4 expression is associated with metastatic behavior. APMIS. 115(7):838-847.

Tsukita S, Furuse M, Itoh M. 2001. Multifunctional strands in tight junctions. Nat Rev MolCellBiol.,2(4):285-93.

Tsukita S, Furuse M. 2000. The structure and function of claudins, cell adhesion molecules at tight junctions. Ann N Y Acad Sci. 915:129-135.

Van Itallie CM, Anderson JM. 2006. Claudins and epithelial paracellular transport. Annu Rev Physiol 68: 403–429.

Van Itallie CM, Holmes J, Bridges A, Gookin JL, Coccaro MR, Proctor W, Colegio OR, Anderson JM. 2008. The density of small tight junction pores varies among cell types and is increased by expression of claudin-2. J Cell Sci. 121(Pt 3):298-305.

C h a p t e r 1 Expression Profile of Claudins in *Mozambique Tilapia* Gastrointestinal Tract

ABSTRACT

Claudins are evolutionary conserved proteins, constituting the main structural and functional component of tight junctions. Claudin proteins play crucial roles in regulation of paracellular spaces in numerous tissues in mammals. In fish, claudins have also been reported to be present in various tissues. Here the subcellular localization of claudin-3 and -4-like proteins in the gastrointestinal tract of both sea and fresh water-acclimated Mozambique tilapia was reported by immunohistochemistry studies. In addition, cldn3c, cldn28a, and cldn30 in the gut of SW and FW-acclimated and salinity challenged tilapia were analyzed using quantitative real-time PCR. Presence of high expression of these claudins at protein and mRNA levels along different segments of the gastrointestinal tract suggests that these claudins participate in regulating epithelial water and solute paracellular permeability in euryhaline tilapia, *Oreochromis mossambicus.*

INTRODUCTION

In nature, a wide number of fish species have adapted to live only either in marine or fresh water; however, few fish species, such as the euryhaline tilapia, *Oreochromis mossambicus*, have developed plasticity to migrate between both aquatic environments during their lifecycle. In these three environments, fishes face different osmotic problems and have to develop different anatomical and physiological osmoregulatory strategies to

maintain a balanced internal environment (reviewed by Marshall and Grosell, 2005). The main osmoregulatory organs are the gills, skin, kidneys, and gastrointestinal tract (GI tract). Epithelial layers in these organs separate sea or fresh water from the blood supply; therefore, these cell layers play a crucial role for osmoregulation, and are organized in a tissue specific manner. Two main routes exist for transportation between the external aquatic environment and the blood supply: transcellular and paracellular pathways. In the transcellular pathway, water and solutes are transported through the epithelial cell layers by simple diffusion or a variety of channels and transporters. On the other hand, spaces between the epithelial cells also allow the transportation of water and ions in a size and charge specific manner and are called paracellular pathways (Gonzalez-Mariscal et al., 2003). Transport via paracellular pathways is regulated by specialized structures called tight junctions (TJs) which are located at the apico-lateral epithelial plasma membranes. The main component of TJs is the transmembrane claudin (cldn) protein family (reviewed by: Van Itallie and Anderson, 2006; Gonzalez-Mariscal et al., 2003).

In this chapter, I focus on the expression of claudin-like proteins and specific claudin mRNA's in the gastrointestinal tract of tilapia challenged to fresh water and sea water.

The Objectives and Significance of the Study:

The properties of transcellular transport (Loretz, 1995; Marshall, 2002; Evans et. al., 2005) and the ultrastructure/electrophysiology of TJs in different epithelia have been well examined in fish (Sardet et al., 1979; Kelly and Wood 2001; Wood et. al, 2002). However, only recently have cldns been identified and shown to be expressed in fish tissues,

including in the gastrointestinal tract of fish (Tipsmark et. al., 2008a; Tipsmark et. al., 2008b; Bagherie-Lachidan et al., 2008; Loh et. al., 2004). Consequently, their roles in epithelial function with respect to osmoregulation are poorly understood. Since cldns are the main components of tight junctions, it is necessary to examine the expression patterns of cldns to understand their functions. Expression patterns can help to reveal the functions of certain cldns in osmoregulation, which are largely unknown. Cldn3 and 4 are prevalent and important TJ proteins along different organs in the GI tract as well as in many other organs in mammals (Rahner et al., 2001; Holmes et al., 2006). The presence of cldn3 was also detected in birds and amphibians (our unpublished data and Haworth et. al., 2005; Brizuela et. al., 2001). Four cldn3 genes (Tncldn3a, Tncldn3b, Tncldn3c, and Tncldn3d) were identified in the euryhaline spotted green puffer fish, *Tetraodon nigroviridis,* and their mRNA distributions in the kidney, skin, gill, and intestine were reported (Bagherie-Lachidan et al., 2008). Changes in Tncldn3 expression in different tissues in response to different environmental salinity have been described (Bagherie-Lachidan et al., 2008). In *Fugu rubripes*, four cldn 3 genes have been identified and tissue specific expression patterns of fu-cldn genes were similar to Tetraodon (Loh et al., 2004; Bagherie-Lachidan et al., 2008). In the present study, it was hypothesized that cldn 3 and 4-like proteins would be widely distributed along different segments of the GI tract of sea and fresh water adapted Mozambique tilapia. Secondly, these proteins would play roles in the osmoregulation of the GI tract epithelia in a tissue-specific manner; consequently, they would be regulated differently in hypoosmotic (fresh water) and hyperosmotic (sea water) conditions to maintain a stable plasma osmolality.

Paracellular pathways are quite complex with respect to permeability for charged and non-charged solutes (Oliveira and Morgado-Diaz, 2007). Some claudin proteins were shown to form charge specific pores for ions (Coleigo et al., 2002; Van Itallie et al., 2001 and 2008). In addition, they may regulate paracellular flux of larger non-charged molecules although the mechanism(s) is not well understood.

Fresh water fishes live in electrolyte-poor environments. Dietary ion uptake is important for a stable ionic homeostasis, and fresh water fish intestine must have a high absorption capacity for ions, mainly Na^+ and Cl^-. Indeed, previous in vitro studies have shown high ionic permeability of fresh water tilapia intestine in both anterior and posterior intestines (Mainoya, 1982; Rawdon and Cornish, 1973). The main route of Na^+ and Cl^- uptake is through transcellular, incorporating various apically located transporters with energy is provided by basolaterally located Na^+ pumps. In this study, it is hypothesized that various claudins would form cation discriminating pores in the paracellular spaces to prevent backflow of ions from plasma to the lumen side of the intestine in fresh water environments. At the same time, since fresh water fish have hydration problems due to dilute surrounding environment, tight junctions would be relatively tighter to prevent water from diffuse paracellularly into the blood. Therefore, the expression of claudins having cation discriminating and sealing potentials would be expected to be higher in fresh water acclimated fish than that of sea water fish intestine. To test these hypotheses, Mozambique tilapia was used as a model organism as the mechanisms governing hydromineral balance has been well studied in this euryhaline fish. They were acclimated into distinct salinity conditions: sea water (SW) and fresh water (FW). Distribution of certain cldns was

screened along the GI tract, and the responsiveness of cldns at both the protein and mRNA levels was examined. The dynamics of TJ remodeling and the properties of epithelial tissue along the GI tract during osmotic challenge are discussed.

LITERATURE REVIEW

Osmoregulation in Sea Water-Acclimated Tilapia GI Tract:

In SW fish, plasma osmolality (300–350 mosmol/kg) is maintained below that of the marine environment (~1000 mosmol/kg). Therefore, fish continuously lose water and gain ions mainly through the large surface area of gill epithelia when held in seawater. The loss in water is restored by drinking to prevent dehydration (Marshall and Grosell, 2005). Therefore, the GI tract plays a direct role in osmoregulation in marine fish through absorption of water. Lost water regained by the gastrointestinal tract is accompanied by an obligatory salt load. This unavoidable, excessive salt is eliminated by its active secretion from the gill and skin epithelia. In SW fish, the esophagus, stomach, and the intestine of the **GI tract** play different osmoregulatory roles.

The esophagus is the first part of the GI tract for which ingested fluid is osmotically processed. The esophageal epithelium displays roughly 6 morphological adaptations between SW and FW-acclimated fish. A relatively higher number of mucosal folding to increase surface area is seen in SW eels (Yamamoto and Hirano, 1978). SW-acclimated European and Japanese eels have columnar epithelia which line the mucosal layer of the esophagus and are different from stratified epithelium of FW adapted fish (Hirano and Mayer-Gostan, 1976; Yamamoto and Hirano, 1978). In SW eel, simple columnar epithelium contains a high number of mitochondria, and includes few mucous cells. Additionally, the presence of intercellular spaces was reported in the simple columnar epithelium (Laurent and Kirsch, 1975). Higher vascularization in the esophageal connective

tissue layer is another morphological difference from FW fish (Yamamoto and Hirano, 1978).

In SW, the esophagus absorbs salt with little passive transport of water (Hirano and Mayer-Gostan, 1976; Parmelee and Renfro, 1983). Absorption of NaCl is driven by basolaterally located Na^+-K^+ ATPases which transport Na^+ towards the blood side in the presence of Cl^- (Parmelee and Renfro, 1983). As a result of salt removal from the ingested fluid, the osmolality of the luminal fluid becomes lower as it reaches into the stomach (~400mOsm), and is almost isotonic to the plasma within the intestine, where fluid absorption occurs (Parmelee and Renfro, 1983).

The intestine of the GI tract is the main area for water absorption in marine fish. It has been proposed that both transcellular and paracellular pathways play roles in water absorption, and this function depends on the presence and absorption of Na^+ and Cl^- ions. The main transporters involved in salt and water absorption in the intestine include basolaterally located Na^+-K^+- ATPase (Na^+ pumps), Cl^- channels, and K^+-Cl^- co-transporters; and apically located two co-transport systems: Na^+-Cl^- (NC) and Na^+-K^+-$2Cl^-$ (NKCC) as well as an anion exchanger $Cl^-/HCO3^-$ (Frizzell et al., 1979; Musch et al., 1982; Grosell et al., 2005; Marshall and Grosell, 2005). Activity of apical NC and NKCC transporters are driven by basolateral Na^+-K^+- ATPases. Na^+-K^+- ATPase pumps intracellular Na^+ into the blood side, which generates an electrochemical gradient favoring apical Na^+, K^+, and Cl^- uptake into the intestinal epithelial cells. Cl^- exits the cell across the basolateral membrane through Cl^- channels and K^+-Cl^- co-transporters (Loretz and Fourtner, 1988, Halm et al., 1985). Water follows these ions by osmosis possibly using both

the paracellular and transcellular pathways (Marshall and Grosell, 2005). Presence of aquaporins was reported in the fish intestine but their functions in water absorption are not known (Marshall and Grosell, 2005; Lignot et al., 2002). Apical Cl^-/HCO_3^- exchangers contribute up to 70% of net Cl^- absorption (Grosell et. al., 2005; Grosell and Genz, 2006). These antiport systems secrete $HCO3^-$ into the lumen and cause the intestinal fluid to be highly alkaline (Wilson et al., 2002). $HCO3^-$ may originate from the hydration reaction of CO_2 by carbonic anhydrase. High HCO_3^- neutralizes the acidification of the lumen from the entry of the gastric chyme, and also facilitates Cl^- and water absorption and $CaCO_3$ formation (Taylor and Grosell, 2006; Taylor et. al., 2007). Mg^{2+} and SO_4^{2-} are the main ions that are found in the intestinal fluid due to preferential absorption of Na^+ and Cl^- in the intestine of SW fish (Mcdonald and Grosell, 2006).

In addition to the GI tract, the gills and kidney have crucial functions for hydromineral balance in SW fish. The SW fish the **kidney** plays an important role for osmoregulation and is adapted to produce an isotonic low volume of urine which is rich in secreted Ca^{2+}, Mg^{2+}, SO_4^{2-} (Beyenbach, 2004). The absorption of water by the kidney mostly depends on the intake of Na^+ and Cl^- ions in SW fish. The majority of this obligatory salt loading is eliminated by the gills. NaCl is actively excreted by the **gill** epithelium.

Osmoregulation in FW-Acclimated Tilapia GI Tract:

In contrast to SW fish, fish face excessive hydration problems due to hypoosmatic surrounding freshwater environment (~1 mOsm). In addition to water gain, they experience ion loss mainly through the large surface area of gill epithelium (Marshall and Grosell,

2005). In contrast to SW fish which actively secrete ions across the gill epithelium, the gill of FW fish actively take up ions from their environment (Marshall and Grosell, 2005). Both chloride and pavement cells are believed to be involved in Na^+ and Cl^- uptake by gills (McCormick, 2001). Cl^- is taken into the chloride cells from the apical side by an exchange with HCO_3^-. This creates an electrical gradient in favor of Na^+ to enter into the chloride cells, and Na^+ ions are transported towards plasma by Na^+-K^+ ATPase (McCormick, 2001). The presence of apical H^+-Na^+ exchanger or Na^+ channels coupled with H^+-ATPase were reported on the apical sides of pavement cells in Mozambique tilapia (Hiroi et al., 1998). Some salts may be taken up by dietary foods and absorbed by the gut. The kidney produces dilute, large volumes of dilute urine to cope with excessive hydration (Nishimura and Imai, 1982; Marshall and Grosell, 2005).

As with SW adapted fish, different parts of **the GI tract** of FW fish have differential osmoregulatory functions. The main osmoregulatory role of the GI tract of the fresh water fish is to form a barrier against surrounding water to minimize its diffusion into the blood stream while maximizing absorption of essential ions from the diet (Smith et. al., 1989). This change in osmoregulation is regulated by modification of the morphology and structure of the GI tract epithelium. As opposed to SW fish, in FW eel, the esophagus has stratified epithelium including numerous mucous cells (Yamamoto and Hirano, 1978). These mucous releasing cells are located mainly in the middle zone of the esophageal epithelium. Ribosome rich cells and filament rich cells are the other members of the epithelial layer. Filament rich cells are located in both the outermost and basal parts of the epithelium layer and are tightly connected to each other, probably limiting diffusion of

water through the epithelium (Yamamoto and Hirano, 1978). In contrast to these chronic adaptations, acute modifications such as changes in the activity and permeability of membrane channels and transporters have been reported (Tipsmark et al., 2008b).

Differential regulation of cell proliferation and apoptosis play an important role in the alteration in epithelial morphology. In addition, changes in number and activity of membrane channels and transporters, such as Na^+-K^+ ATPase, alter the membrane permeability for ions and water during salinity adaptation (Takahashi et. al., 2007). Hormonal regulation of both epithelial morphology and physiology has been reported. While glucocorticoids (cortisol) are essentially associated with SW adaptation, prolactin was proposed to be especially important for FW adaptation (Mccormick, 2001; Sakamoto et. al., 2005). Prolactin is one of the key hormones for FW adaptation in fish (McMormick, 2001; Sakamoto et. al., 2005). Prolactin receptor expression was detected in the GI tract epithelium of Nile tilapia (Sandra et. al., 2000). In FW acclimation, prolactin expression was increased and its localization was detected in the proliferating cells in the esophagus epithelium of Mozambique tilapia (Takahashi et. al., 2007). In Mozambique tilapia, glucocorticoid and prolactin signals were suggested to be involved in regulating esophageal epithelial cell turnover during salinity acclimation (Takahashi et al., 2006). Some cortisol-stimulated adaptations in SW fish are: increased water and ion uptake in the gut, increased Na^+-K^+ ATPase activity and Na^+ excretion at the gills, and increased water permeability of the urinary bladder (Marshall and Grosell, 2005). In addition to cortisol and prolactin, growth hormone and insulin like growth factor 1 (IGF-1) may promote salinity adaptation (Tipsmark et al., 2008b; Madsen et al., 2007; Fiess et al., 2007; Mccormick, 2001). To

conclude, differential organization of the GI tract, gill, kidney and urinary bladder epithelia is accompanied by increased ions and water uptake in SW fish relative to FW fish (Takahashi et. al., 2007; McCormick, 2001).

Tight Junctions and Claudins in Fish:

Tight junctions are located at the apico-lateral sides of numerous epithelia including GI tract in fish (Anderson and Van Itallie, 2008; Bagherie-Lachidan et al., 2008). Tight junctions regulate paracellular transport and they play a crucial role for osmoregulation by complementing the transcellular pathways whose properties have been identified in detail in various epithelia (Evans et al., 2005). The claudin transmembrane protein family is believed to be the main structural and functional component of TJs (Furuse et al., 1998; Furuse et al., 1996; Krause et al., 2008). They have 4 transmembrane domains; their short amino (2-6 aminoacid residues) and carboxyl termini are located in the cytoplasm. They have two extracellular loops. The first extracellular loop of claudins forms bridges between the cells. Claudins form size and charge specific pores in the paracellular spaces (Krause et al., 2008; Van Itallie et. al., 2008; Gonzalez-Mariscal et. al., 2003; Colegio et al., 2002). The net charge of the first extracellular loops is believed to determine charge specificity (Krause et al., 2008; Van Itallie et. al., 2003; Colegio et. al., 2003).

Although the properties of the transcellular transport (Loretz 1995 and Marshall 2002) and the ultrastructure/electrophysiology of TJs in different epithelia have been well examined in fish (Sardet et al., 1979; Kelly and Wood 2001; Wood et. al, 2002), cldns have only recently been identified and shown to be expressed in various tissues of fish (Tipsmark et. al., 2008a; Tipsmark et. al., 2008b; Loh et. al., 2004). Whereas about 24 claudin

members have been identified in mammals (Furuse and Tsukita, 2006; Van Itallie and Anderson, 2006), in fish this number is as high as 57 due to tandem gene duplication and/or possible whole genome duplication events probably in response to the diversity of aquatic environments (Loh et al, 2004; Christoffels et al., 2004). In *Fugu rubripes,* gene duplication events were suggested to produce 17 genes that are close to the mammalian cldn3 and 4 (Loh et al., 2004).

Transepithelial resistance (TER) of cultured gill epithelium was compared in SW and FW fish and reported that FW gill epithelium has significantly higher 600–10,000 cm^2 TER than that of SW fish 200–300 cm^2 (Wood et al., 2002). After SW transfer, ultrastructural changes in the TJs were detected between chloride cells and accessory cells in the gill epithelium (Marshall et al., 1997). Presence of leaky TJs between these two cell types play an important role in Na+ secretion (Sardet et al., 1979), providing higher ionic permeability in the gill of SW eel than in FW eel gill (Isaia and Hirano, 1976).

In our laboratory the presence of cldn 3 and cldn 4-like proteins was shown in the tilapia gill epithelium (Fig. 1.1) and their expression at the mRNA and protein levels were sensitive to environmental salinity (Tipsmark et. al., 2008a). In the gill of FW fish, cldn 3 staining was localized mainly in the deeper cell layers in the filament but also in pillar cells on the lamellae (Fig. 1.1B). In the SW gill, cldn 3 antibody staining was closer to the apical side of the filament (Fig. 1.1E).

On immunoblots of gill protein preparations, the antibody directed against mammalian claudin 3 revealed one single band with apparent molecular mass of 18 kDa (Fig. 1.1).

In the FW gill, the claudin 4 antibody stained epithelial cells in the filament but also some cells on the lamellae, including pillar cells (Fig. 1.2B). In the SW gill, claudin 4 antibody staining was substantially weaker, found mostly in a thin layer on the apical side of epithelial cells of the filament (Fig. 1.2E); no staining was seen in pillar cells (Fig. 2E). Negative staining was prepared by replacing primary antibody with normal serum, and this abolished the reaction (Figs. 1.1 and 1.2, A and D). Binding of both antibodies to gill cells was blocked when preabsorbed with their respective blocking peptides, which shows that staining was specific for both claudin antibodies (Figs. 1.1 and 1.2, C and F).

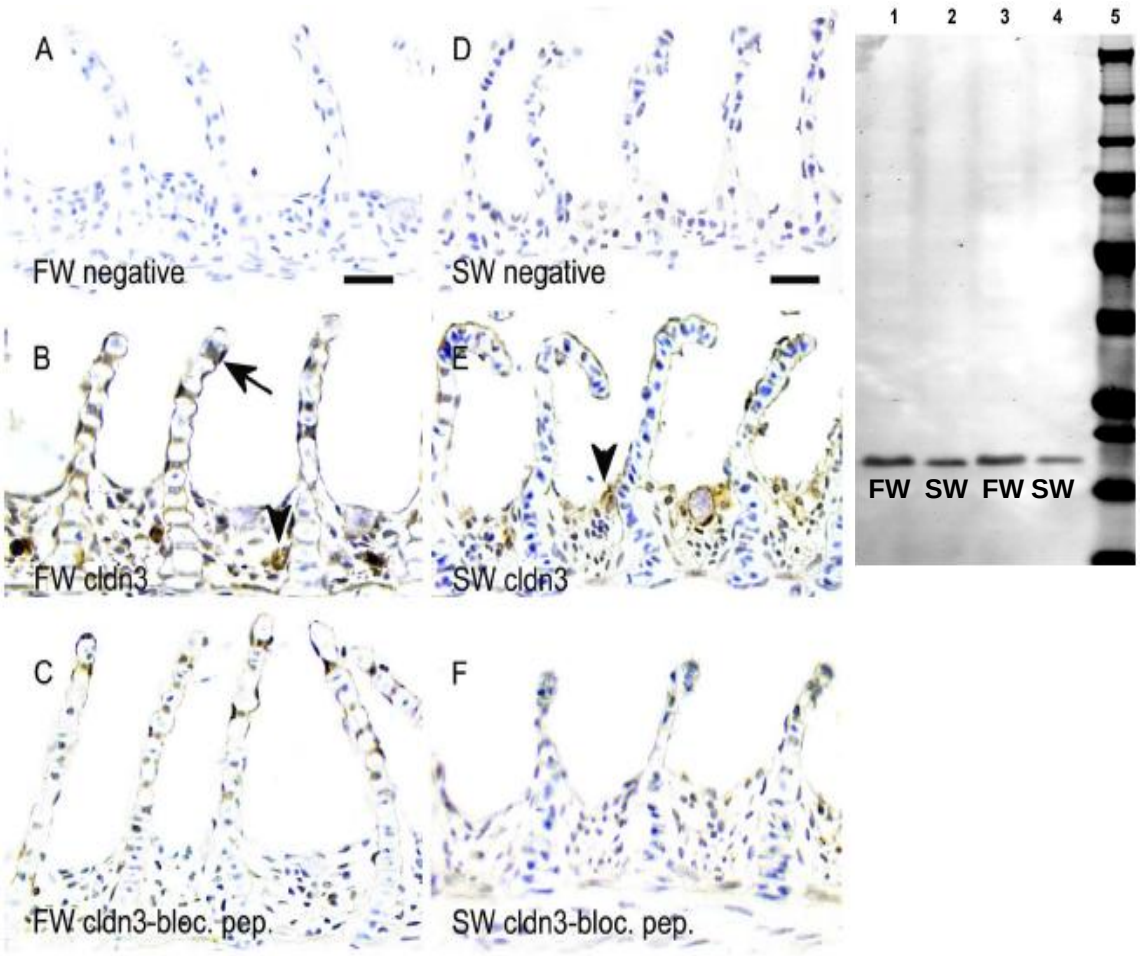


Figure 1.1: Claudin 3-like protein immunostaining (left) and western blotting (right) of SW and FW-acclimated fish gill epithelium.

Left: gill sections (6 μm) from FW (A–C) and SW tilapia (D–F) were immunostained with the cldn 3 antibody. Staining was specific, as verified by the lack of staining with normal serum (A and D) and when the antibody was preabsorbed with blocking peptide (cldn3-bloc. pep.; C and F). Sections were counterstained with hematoxylin. The arrowheads and arrow indicate examples of localized staining in the filament and on the lamellae, respectively. Bars: 20 μm.

Right: freshwater (FW; lanes 1 and 3) and seawater preparations (SW; lanes 2 and 4) were probed with mouse claudin 3 antibody. Molecular mass standards are displayed in lane 5 in A and B (from top to bottom: 250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kDa).

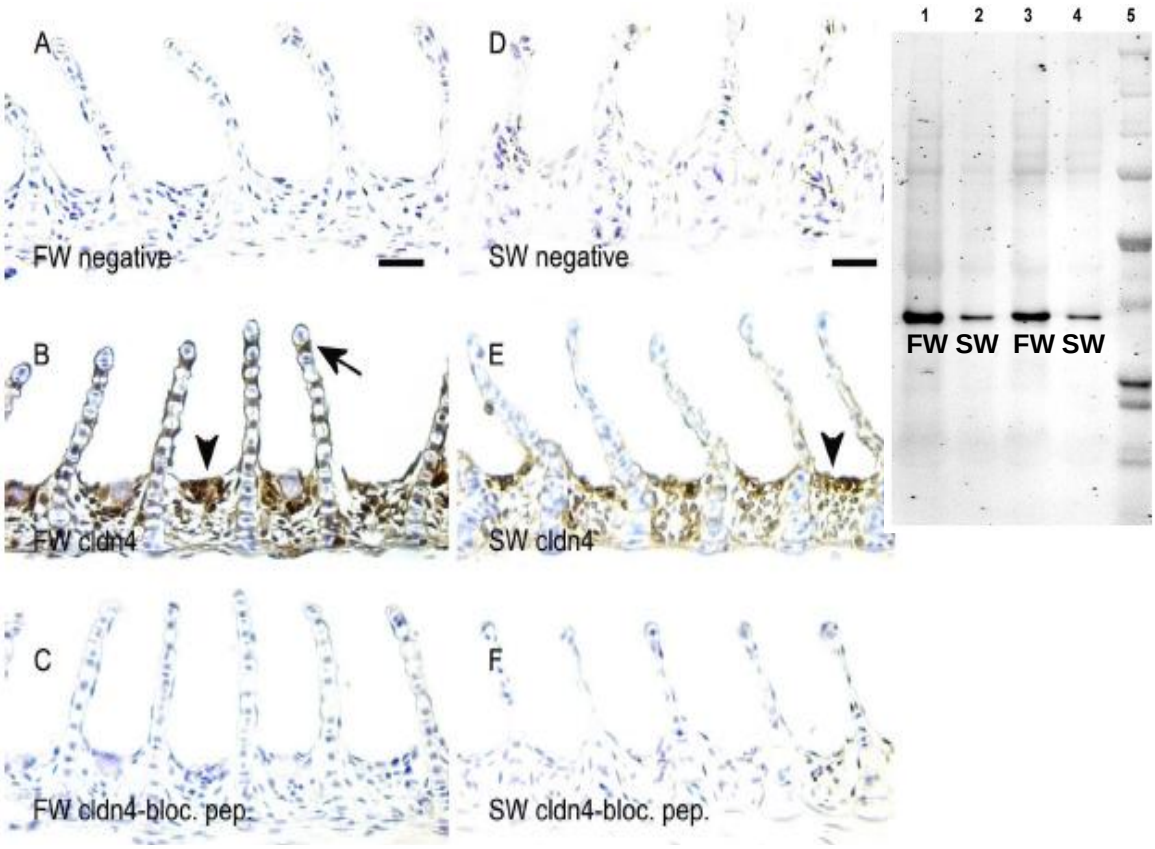


Figure 1.2: Claudin 4-like protein immunostaining (left) and western blotting (right) analyses in the SW and FW-acclimated fish gill epithelium.
Left: Gill sections (6 μm) from FW (A–C) and SW tilapia (D–F), immunostained with the claudin 4 antibody (cldn4). Staining was specific, as verified by the lack of staining with normal serum (A and D) and when the antibody was preabsorbed with blocking peptide (cldn4-bloc. pep.; C and F). Sections were counterstained with hematoxylin. The arrowheads and arrow indicate examples of localized staining in the filament and on the lamellae, respectively. Bar, 20 μm.
Right: FW (lanes 1 and 3) and SW preparations (lanes 2 and 4) were probed with claudin 4 antibody. Molecular mass standards are displayed in lane 5 in A and B (from top to bottom: 250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kDa).

Four cldn3 genes (Tncldn3a, Tncldn3b, Tncldn3c, and Tncldn3d) were characterized in the euryhaline spotted green puffer fish *Tetraodon nigroviridis* and their mRNA distributions in the kidney, skin, gill, and intestine were reported (Bagherie-Lachidan et al., 2008). Changes in Tncldn3 expression in different tissues in response to

different environmental salinity conditions were also described (Bagherie-Lachidan et al., 2008). While renal and intestinal tissues express all four Tncldn3 isoforms, the gills and skin express only Tncldn3a and Tncldn3c. In *Tetraodon* intestine, the expression levels of Tncldn3a, Tncldn3b and a lesser extent Tncldn3d were higher in FW-acclimated fish than SW-acclimated fish intestine. The expression levels of Tncldn3c were similar between SW and FW acclimated- *Tetraodon* intestines. Like *Tetraodon*, in *Fugu rubripes*, four cldn 3 genes have been identified. The tissue specific expression patterns of fu-cldn genes were reported to be similar to Tetraodon and all four cldn genes were present in the intestine (Loh et al., 2004). In *Dicentrarchus labrax*, cldn3 gene expression was also detected in the intestine, and preliminary evidence suggests it may change depending on environmental conditions (Boutet et al., 2006).

MATERIALS AND METHODS

Animals:

One group of adult male tilapia was maintained in FW at 1-2 ppt salinity, another group was incubated in SW (25 ppt salinity). They were acclimated to the respective salinities for a minimum 30 days before starting transfer experiments.

Sampling:

Fish were anaesthetized in buffered MS-222. The fish were killed by spinal section, and the GI tract was carefully removed. Tissue pieces approximately 1cm from the esophagus, stomach, and posterior intestine were taken. For immunohistochemistry analyses, pieces were fixed in fresh, buffered paraformaldehyde (4%) for 4 h. For real-time

PCR analyses, tissue pieces from posterior intestine were directly frozen in liquid nitrogen and stored at 80°C until further processing.

Immunohistochemistry:

Fixed tissues were embedded in OCT (Tissue-Tek OCT compound-Sakura) and frozen in liquid nitrogen. Frozen tissue blocks were sectioned at 7μm by a cryostat vibratome (Bright OTF5000 – Bright Instrument Co Ltd.) at -16^{o}C.

For antigen retrieval, slides were boiled in citrate buffer (10 mM sodium citrate and 0.05% Tween 20, pH 6.0) for 40 min at 95°C. Endogenous peroxidase activity was suppressed by treatment with hydrogen peroxide. Subsequently, blocking serum was applied for 1 h at room temperature (Vectastain kit; Vector Laboratories, Burlingame, CA). Sections were incubated with polyclonal claudin 3 and monoclonal claudin 4 antibodies (Zymed, San Francisco, CA, USA) at 1:200 dilutions in PBS (in mM: 137 NaCl, 3 KCl, 10 Na_2HPO_4, and 2 KH_2PO_4, pH 7.4) for 16 h at 4°C in a closed and moistened chamber. Claudin 3 and 4 antibodies are raised against a 22 amino acid peptide sequence derived from the C terminal region of mouse claudin 3 and human claudin 4, respectively. For negative controls, duplicate sections were treated with normal serum for the same time and temperature. After 16 h, diluted biotinylated universal IgG (Vectastain kit; Vector Laboratories) was applied and slides were incubated for 60 min at room temperature. Vectastain ABC reagent was then added for 60 min at room temperature. To visualize the claudin proteins, 3,3-diaminobenzidine (DAB; Sigma) was prepared according to the

manufacturer's instructions, and it was applied to sections for 3 min. The DAB reaction was terminated by dipping the slides into water. Sections were counterstained with hematoxylin.

Microscopy:

Light microscopy was performed with an Olympus compound microscope, using 10x, 20x, 40x and 100x (oil) objectives. Images were captured using a Sony digital video camera and processed using Adobe Photoshop 7.0.

Total RNA Extraction:

Pieces from posterior intestine were homogenized using a hand homogenizer. Total RNA of tilapia posterior intestine was extracted by TRI reagent (Molecular Research Center, Cincinnati, OH). Concentration and purity of the total RNA samples were measured using a Nanodrop ND-1000 spectrophotometer. The quality of the total RNA was further analyzed by 1.5% agarose gel electrophoresis. Only total RNA with sufficient purity (A260/280>1.8) was used for further analyses. TURBO DNase (Ambion, Austin, TX) was used to remove genomic DNA contamination according to the manufacturer instructions. Reverse transcription was performed to synthesize cDNA using High Capacity cDNA Reverse Transcription Kit according to the manufacturer instructions (Applied Biosystems, Foster City, CA, USA) in a thermal cycler.

Quantitative Real-Time PCR Analysis:

To investigate the expression of cldns during development, absolute SYBR green real-time PCR analysis was used as previously described by Tipsmark et al. (2008). Reactions consisted of 2X Brilliant SYBR green QPCR master mix (Stratagene), forward

and reverse primers at 1.5μM, and 2.5ng/ml cDNA sample. The list of the primers and the expected sizes of produced amplicons are shown in Table 1.1. A real-time PCR protocol at 50ºC for 2 min, 95ºC for 10 min followed by 40 cycles of 95ºC for 15 sec and 60ºC for 1 min was used in ABI 7900 HT Sequence Detection System (Applied Biosystems). Cycle threshold (Ct) values were determined in triplicate for all experimental samples. Ct values were transformed using a standard curve of diluted plasmid containing the claudin coding sequence with Ct values (R^2 = 0.93– 0.99). All mRNA data were normalized to total RNA concentration (Bustin, 2000; Picha et al, 2006) and are presented as "DNA copy number per nanogram total RNA". Primer specificity was assessed by melting curve analyses in addition to inspection of real-time PCR products using 4% agarose gel electrophoresis. To test if the real-time PCR was free of DNA contamination and if reagents are free of nucleic acid contamination "none amplicon control" and "none reverse transcriptase control" reactions performed. Two-way factorial ANOVA statistical analyses were performed using the SAS Software.

Table 1.1: The list of the primers, annealing temperatures of primers, and the expected sizes of produced amplicons.

Gene	Primer Name	Sequence (5' to 3')	Tm	Amplicon Size
Cldn28a	Cldn28aQF3	CTC CTG CCC ACC CAA AGA	58	53 bp
	Cldn28aQR3	CTG GCA CCG CCG TAC TTC	59	
Cldn30	Cldn30QF3	GGG AGC TTC ACT GTT CAT TGG	58	90 bp
	Cldn30QR3	GGA GCT GAG TAC TTG GCA GAG TAG T	58	
Cldn3c	Cldn3cQF3	TGG GTG TCA TGA TCT CCG TAG TC	60	52 bp
	Cldn3cQR3	TCA CTT TGG CTT TAC TTC CTT CCT	59	

RESULTS

Expression of claudin-like proteins along the gastrointestinal tract of SW and FW-acclimated fish:

The claudin protein family is evolutionary conserved, and shows high sequence similarity between different species. The availability of commercially available fish antibodies against claudin proteins is lacking. Nevertheless, the specificity of mammalian claudin 3 and 4 antibodies in the tilapia gill has been previously tested (Tipsmark et al., 2008a). Among various claudins tested, only rabbit-anti claudin 3 and mouse-anti claudin 4 antibodies showed a single band in the western blots (Tipsmark et al., 2008a). In addition, incubation of antibodies with corresponding blocking peptide washed the immunostaining away. Thirdly, the locations of claudin 3 and 4 immunostaining were specific and present previously expected locations in the gill epithelium. These two antibodies were also used to determine localization of claudin 3 and 4 in the GI tract of tilapia. Throughout the GI tract, the staining of these proteins was mainly detected in the epithelia.

Expression of claudin 3-like protein in the GI tract epithelia of tilapia:

Consistent with some previous reports in Japanese eel (Yamomato and Hirano, 1978), sea and fresh water-acclimated tilapia **esophagus** epithelia display morphological differences from each other. In SW Mozambique tilapia, the mucosal layer of SW fish esophagus seems to have more folding than FW tilapia esophagus. Relative to SW fish, the epithelial cells of the esophagus of FW fish seems more firmly connected to each other on their apical sides. In both SW and FW tilapia esophagus, mucus secreting cells appear to be present. Claudin 3-like protein is present in the **esophagus** epithelia of both SW and FW

fish (Fig. 1.3A and A'). No drastic detectible intensity difference in cldn 3-like staining between SW and FW esophagus was observed (Fig. 1.3B and B'). In SW fish esophagus, cldn 3-like staining is mainly localized between columnar epithelial cells, mucous secreting cells, and filament rich cells, and with no difference between apical and basal sides of the epithelial cells (Fig. 1.3C). In FW fish esophagus, cldn 3-like staining is between the epithelial cells; however, apical sides of the cells are stained stronger than the basal sides of the cells (Fig. 1.3C'). Negative controls from SW and FW fish esophagus are completely free of cldn-3 immunostaining (Figs. 1.3D and D').

The surface of the **stomach** is covered by a simple columnar epithelium. In SW fish stomach, the mucosal surface is not flat and cells seem relatively loosely packed (Fig. 1.4A). On the other hand, in FW fish stomach, the apical sides of the cells seem tightly connected and form an even surface (Fig. 1.4A'). On the surface of mucosal layer, mucous secreting cells form gland-like structures called gastric pits. The abundance of these pits is similar between the SW and FW fish stomach (Figs. 1.4A, A' and C, C'). In both SW and FW fish stomach, staining is in the apico-lateral sides (tight junctional regions) of the simple columnar cells (arrowheads in Figs. 1.4B and B'). In SW fish stomach, cldn-3 staining is mainly between the epithelial cells at the junctional areas (arrowhead in Fig. 1.4B). Weak expression of cldn 3-like immunostaining in the simple tubular gastric glands beneath the gastric pits in SW fish was observed (Fig. 1.4B). In the FW fish, like in the SW fish, staining is mainly between the epithelial cells at the junctional areas (Fig. 1.4B'); in addition, the apical side of the epithelial layer has cldn 3-like staining (arrowhead in Fig. 1.4B'). No detectible staining in the tubular gastric glands beneath the gastric pits was

observed (Fig. 1.4B'). Negative controls from SW and FW fish stomachs are free of cldn-3 immunostaining (Figs. 1.4C and C'). For both SW and FW fish, cldn 3-like staining is strictly in the tight junctional areas in the epithelial layer in the **posterior intestine** (Figs. 1.5A, A' and B, B'). No detectible staining gradient along crypt-to-villus axis was observed (Figs. 1.5A and A'). Likewise, no detectible difference in cldn-3 like staining intensity was observed between SW and FW tilapia posterior intestine.

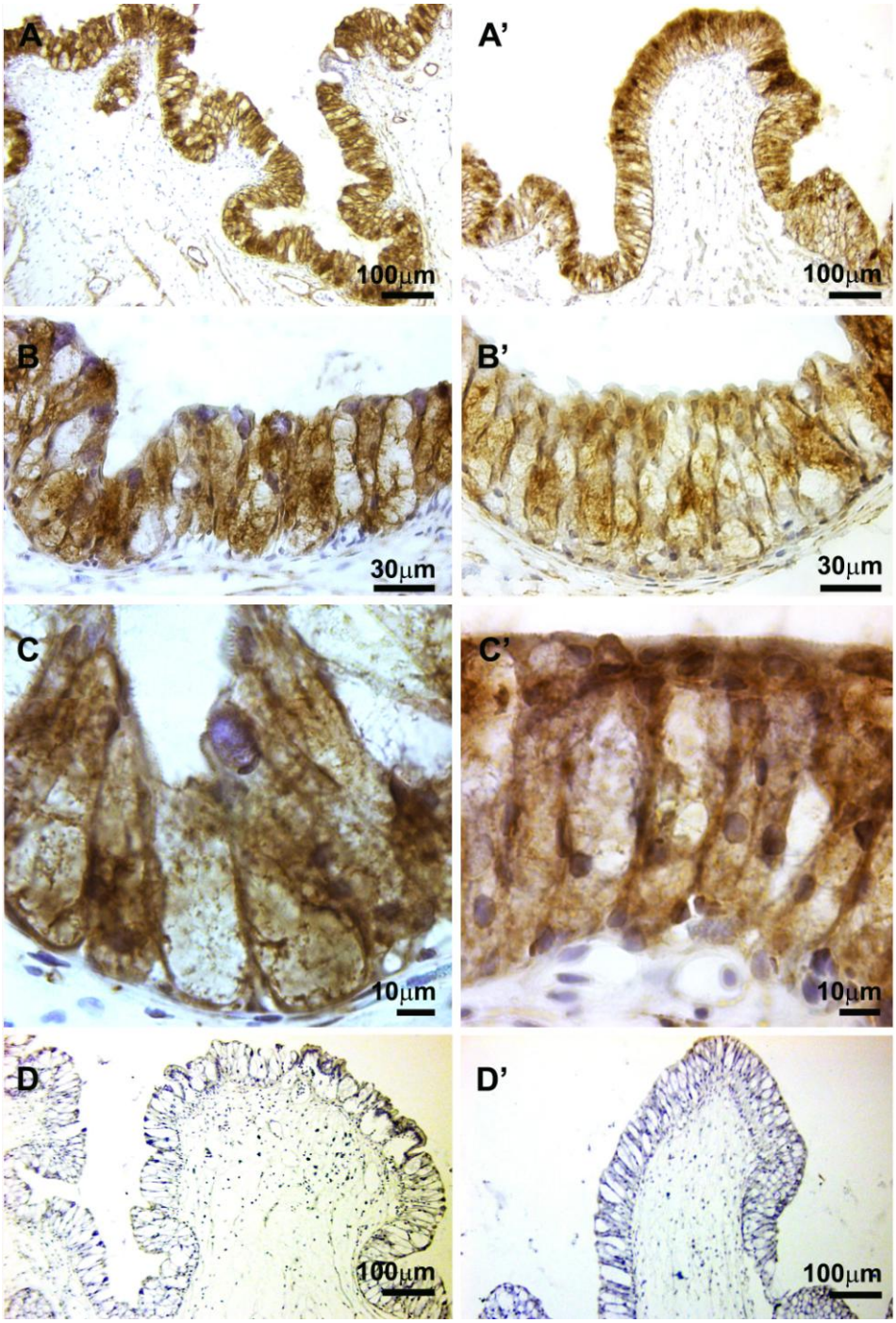


Figure 1.3: Cldn 3-like immunostaining in SW and FW-acclimated tilapia esophagus. Brown color staining shows the localization of the protein. A, B, C and D are from SW-acclimated tilapia; A', B', C' and D' from FW-acclimated tilapia. A, A', B, B' and C, C' are cldn 3-like immunostained sections which were counterstained with hemotoxylin. D and D' are negative controls which were stained only with hemotoxylin.

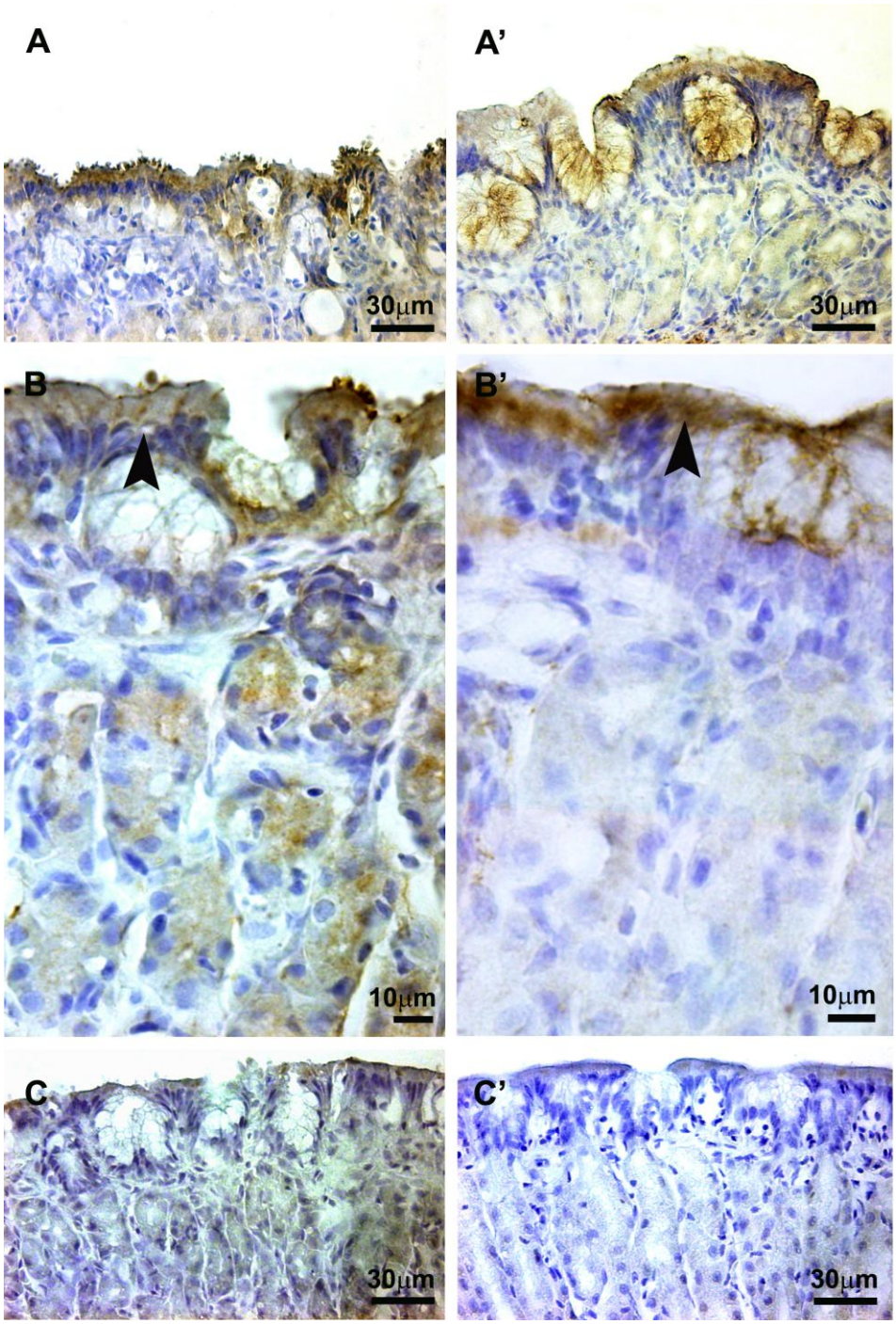


Figure 1.4: Cldn 3-like immunostaining in SW and FW-acclimated tilapia stomach. Brown color staining shows the localization of the protein. A, B, and C are from SW-acclimated tilapia; A', B', and C' from FW-acclimated tilapia. A, A' and B, B' are cldn 3-like immunostained sections which were counterstained with hemotoxylin. Arrowheads indicate tight junctional staining. C, C' are negative controls which were stained only with hemotoxylin.

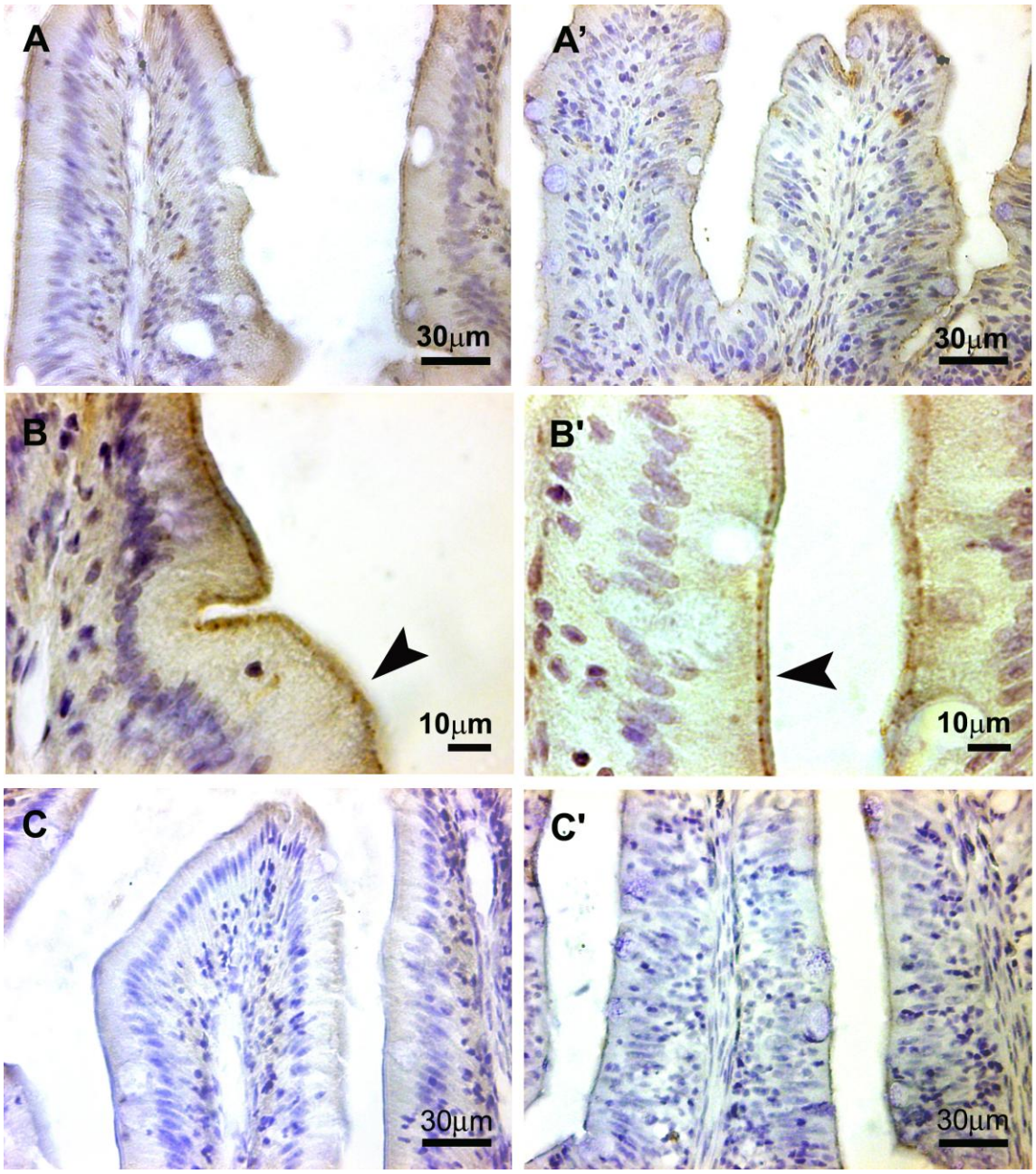


Figure 1.5: Cldn 3-like immunostaining in SW and FW-acclimated tilapia posterior intestine. Brown color staining shows the localization of the protein. A, B, and C are from SW-acclimated tilapia; A', B', and C' from FW-acclimated tilapia. A, A' and B, B' are cldn 3-like immunostained sections which were counterstained with hemotoxylin. Arrowheads indicate tight junctional staining. C, C' are negative controls which were stained only with hemotoxylin.

Expression of claudin 4-like protein in the GI tract epithelia of tilapia:

Claudin 4-like protein is present both in the SW and FW **esophagus** epithelia (Fig. 1.6). Staining in the FW fish esophagus appeared stronger than that observed in the SW fish esophagus (Figs. 1.6A and A', respectively). In SW and FW fish esophagus, cldn 4-like staining was present in both the apical and basal sides of the epithelial cells (Figs. 1.6B and B', respectively). At the apical sides, staining is detected between epithelial cells. When it appears stronger in FW than in SW fish (Figs. 1.6B and B'). Negative controls from SW and FW fish esophagus are free of cldn-4 immunostaining (Figs. 1.6C, C' and D, D'). Claudin-4-like protein is present in the SW and FW tilapia **stomach** (Fig. 1.7). Claudin-4-like protein staining is present between pits (Figs. 1.7B and B'). The intensity of the staining appears higher in FW than SW fish stomach (Figs. 1.7C and C'). In the mucosa, staining is also present in the simple tubular gastric glands beneath the gastric pits (Figs. 1.7B and B'). These gastric glands are formed by two main cell types: chief and parietal cells. These cells can be found in any level of the mucosa, while parietal cells are predominant in the middle zone, chief cells are prevalent in the deeper zone, just above the muscularis mucosae layer. In SW fish stomach, cldn4-like staining is intense in the deeper zone where chief cells are common. On the other hand, staining is more or less homogenous throughout the mucosa of the stomach of FW fish, and staining is detected mostly between the cells (Fig. 1.7B'). Negative controls from SW and FW fish stomachs are completely free of cldn-4 immunostaining (Figs. 1.7 D and D').

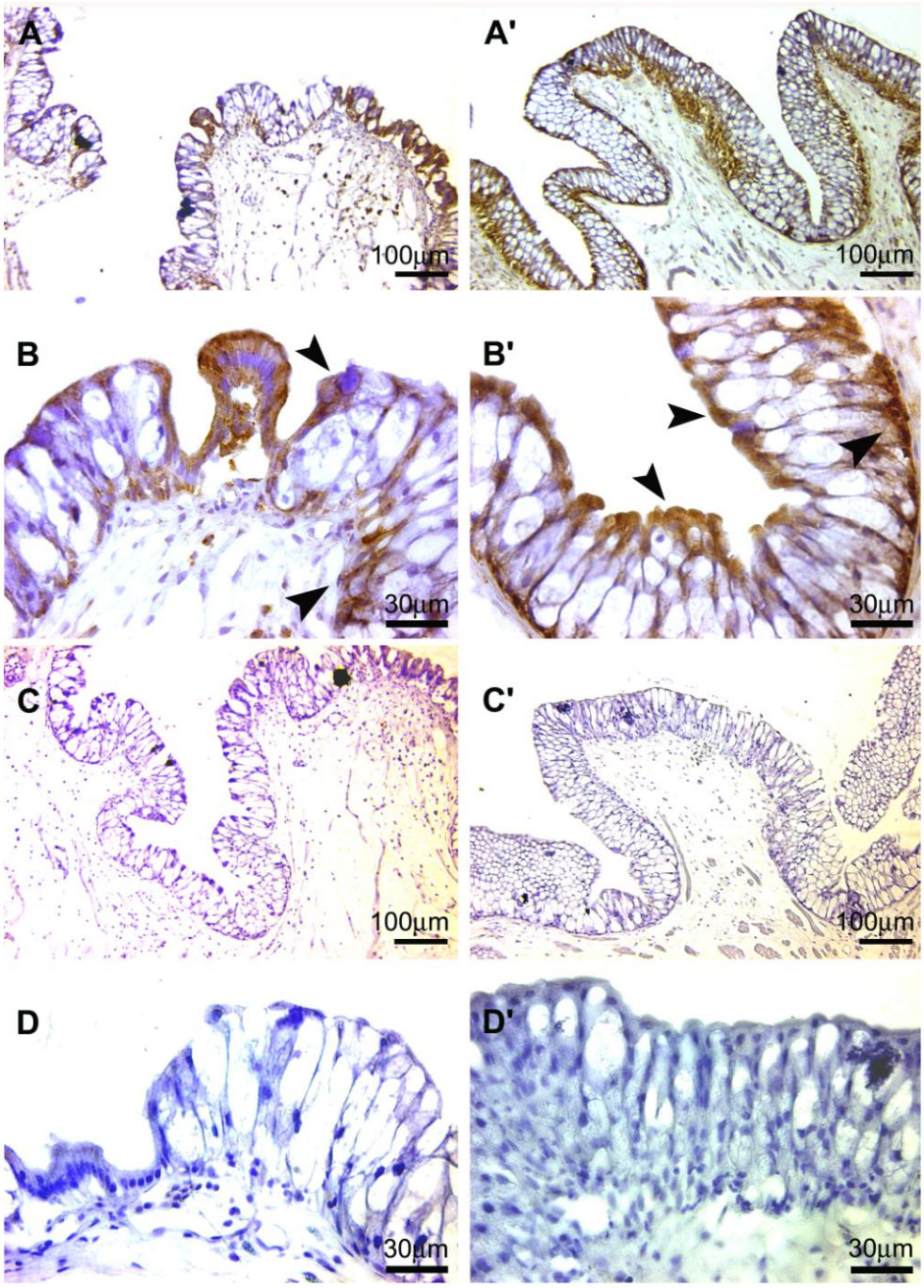


Figure 1.6: Cldn 4-like immunostaining in SW and FW-acclimated tilapia esophagus. Brown color staining shows the localization of the protein. A, B, C, and D are from SW acclimated tilapia; A', B', C', and D' from FW acclimated tilapia. A, A' and B, B' are cldn 4-like immunostained sections. Arrowheads show apical and basal epithelial staining. Immunostained sections were counterstained with nucleus hemotoxylin. C, C' and D, D' are negative controls which were stained only with hemotoxylin.

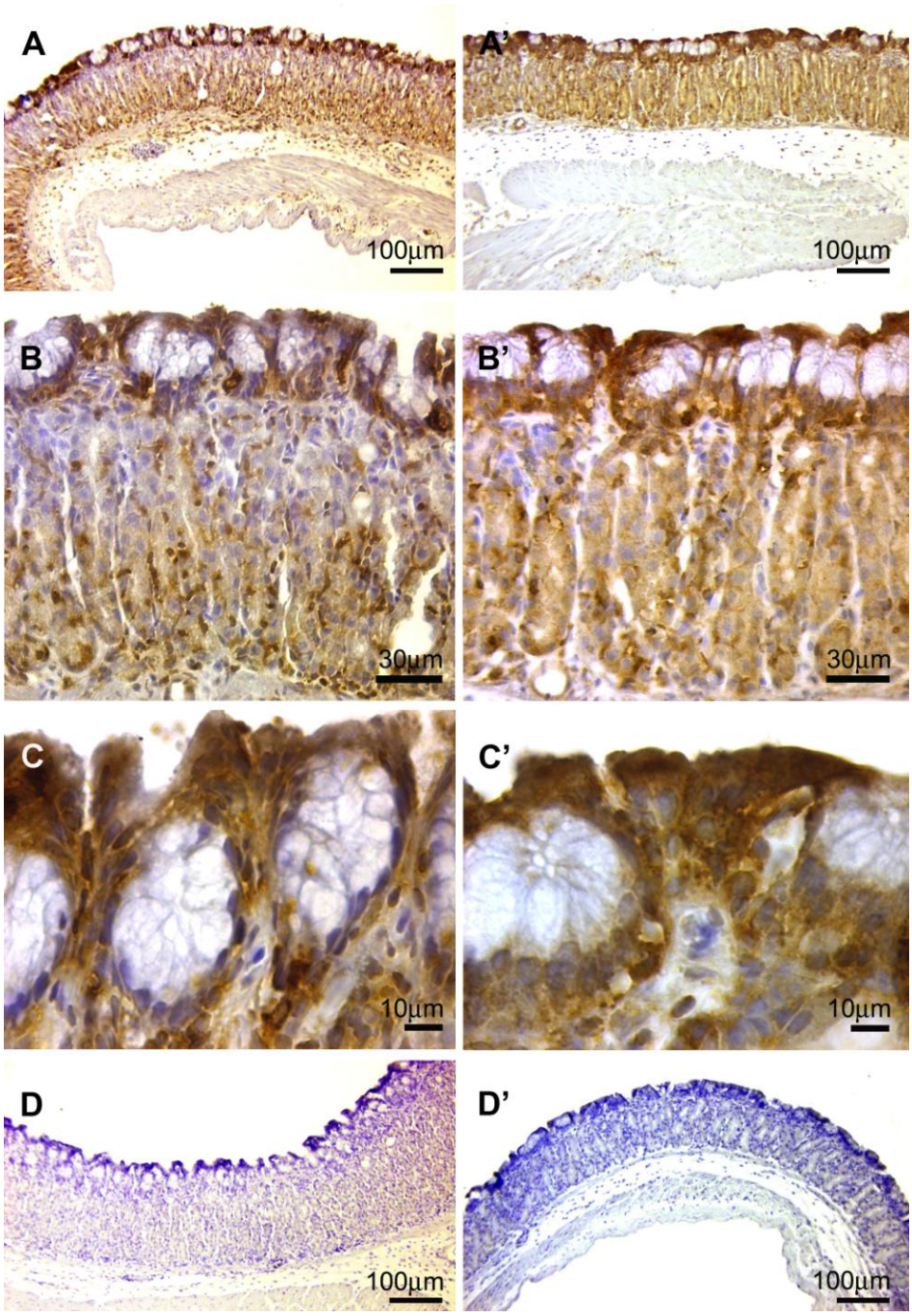


Figure 1.7: Cldn 4-like immunostaining in SW and FW-acclimated tilapia stomach. Brown color staining shows the localization of the protein. A, B, C, and D are from SW acclimated tilapia; A', B', C', and D' from FW acclimated tilapia. A-C and A'-C' are cldn 4-like immunostained sections. Immunostained sections were counterstained with nucleus hemotoxylin. D and D' are negative controls which were stained only with hemotoxylin.

Claudin-4-like protein immunostaining is present in the SW and FW tilapia **posterior intestine** (Fig. 1.8). With respect to morphology of intestine, simple columnar epithelium covers the surface of the mucous layer and primarily consists of enterocytes and goblet cells for both the SW and FW tilapia intestine. The only obvious difference between the SW and FW tilapia intestine is the number of goblet cells. In FW fish, the number of goblet cells is markedly higher in FW fish than SW fish posterior intestine (Figs. 1.8A, A' and C, C'). For both SW and FW fish, cldn 4-like staining is mainly in the apical side of the epithelial layer (Figs. 1.8A, A' and B, B'). No detectible staining gradient along crypt-to-villus axis was observed (Figs. 1.8A and A'). Staining is at the apical side and in the junctional areas between the epithelial cells (Figs. 1.8B and B'). In addition, cldn 4-like staining is detected in the basal sides of the cells in the lower half of the villi in both FW and SW fish intestine (Figs. 1.8A and A').

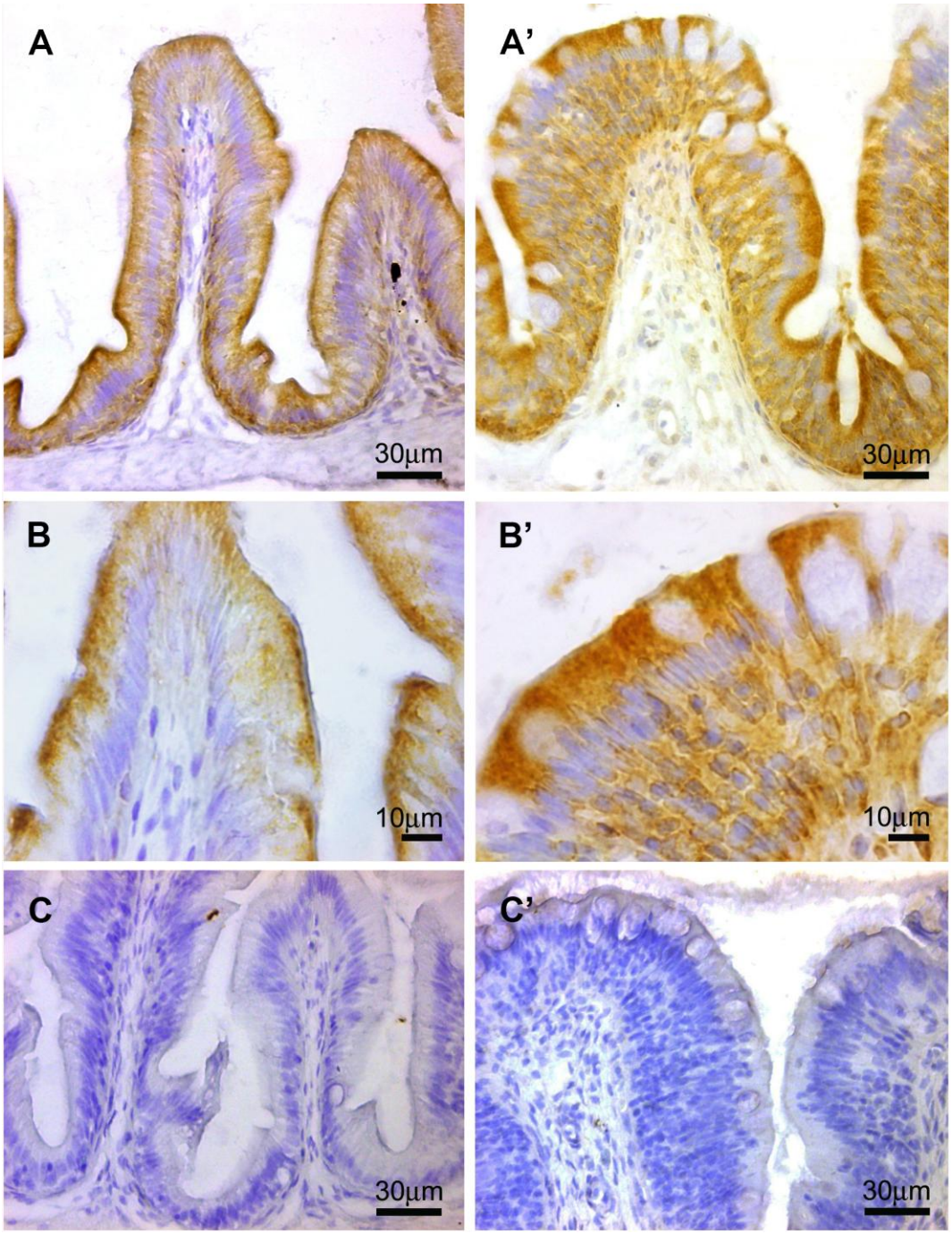


Figure 1.8: Cldn 4-like immunostaining in SW and FW-acclimated tilapia intestine. Brown color staining shows the localization of the protein. A-C are from SW acclimated tilapia; A'-C' from FW acclimated tilapia. A, A' and B, B' are cldn 4-like immunostained sections. Immunostained sections were counterstained with nucleus hemotoxylin. C and C' are negative controls which were stained only with hemotoxylin.

Gene Expression of Claudins in the SW and FW Tilapia GI Tract

Tissue Distribution of Claudin mRNAs in the SW and FW-Acclimated Tilapia GI Tract:

The expression levels of claudin3c, claudin28a, and claudin30 mRNA in the esophagus, stomach, and anterior and posterior intestine of 30-day SW and FW-acclimated tilapia were investigated. The expression levels of these claudins were considerably higher in the esophagus than other three segments of the GI tract. All three claudins shared a common gross expression pattern in individual segments between SW and FW fish gut.

Cldn3c expression was present in all segments of the GI tract examined. No statistically significant differences in cldn3c levels between any segments of SW and FW fish GI tract was observed, although expression levels appear higher in the esophagus and posterior intestine of SW than FW fish. In the stomach and anterior intestine, cldn3c expressions were higher in FW than SW fish, albeit the difference was not statistically significant (Fig. 1.9).

Cldn28a expression was also present along the tilapia gut, but its mRNA levels were extremely low, particularly in the posterior intestine of both FW and SW fish. Only in the stomach was expression significantly higher (3.1 folds) in SW than in FW fish. In the esophagus, anterior and posterior intestines, cldn28a levels in FW and SW fish were similar (Fig. 1.10).

As with cldn28a and cldn3c, cldn30 mRNA levels were highest in the esophagus. No significant difference in cldn30 levels between any segments of SW and FW fish gut was observed (Fig. 1.11).

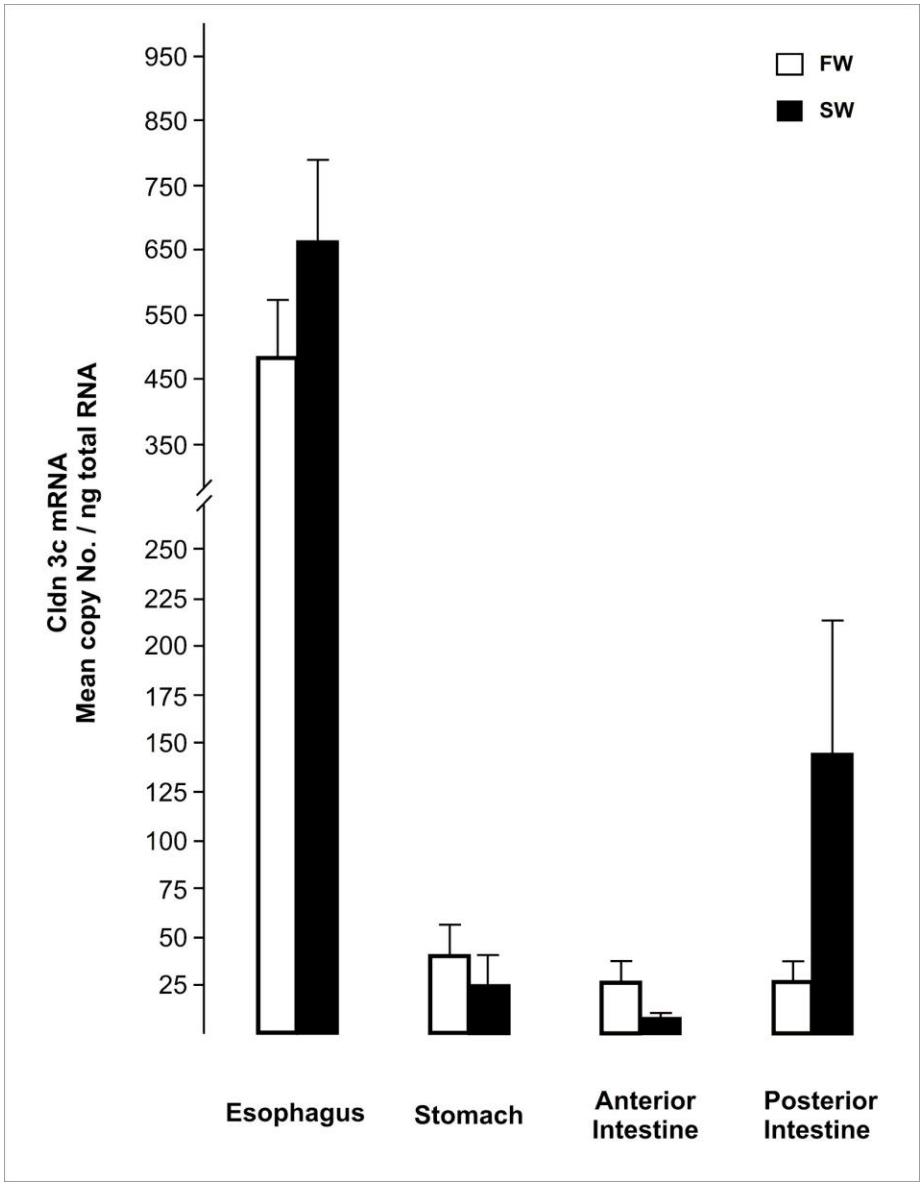


Figure 1.9: Cldn3c mRNA expression along the GI tract of SW and FW tilapia. The expression of cldn3c is highest in the esophagus among four segments examined. There is no statistical difference in cldn3c mRNA levels between SW and FW fish GI tract segments. Values are mean +/- SEM, n= 6 tilapia per each treatment group.

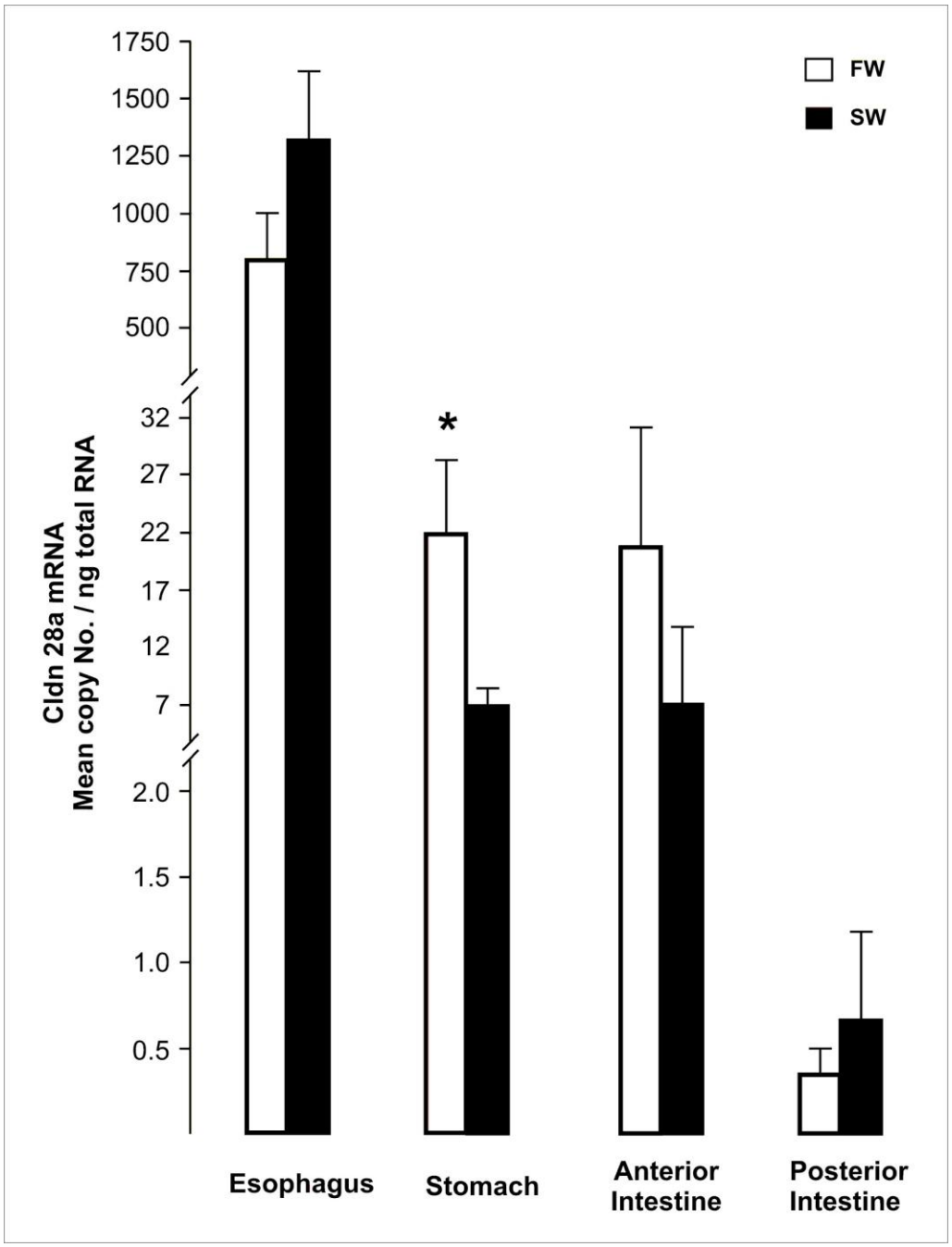


Figure 1.10: Cldn28a mRNA expression along the GI tract of SW and FW-acclimated tilapia. The expression of cldn28a is the highest in the esophagus among four segments examined. In stomach the expression of cldn28a was significantly higher in FW than SW fish. "*" indicates statistical difference between SW and FW fish (*: $p > 0.05$). Values are mean +/- SEM, n = 6 tilapia per each treatment group.

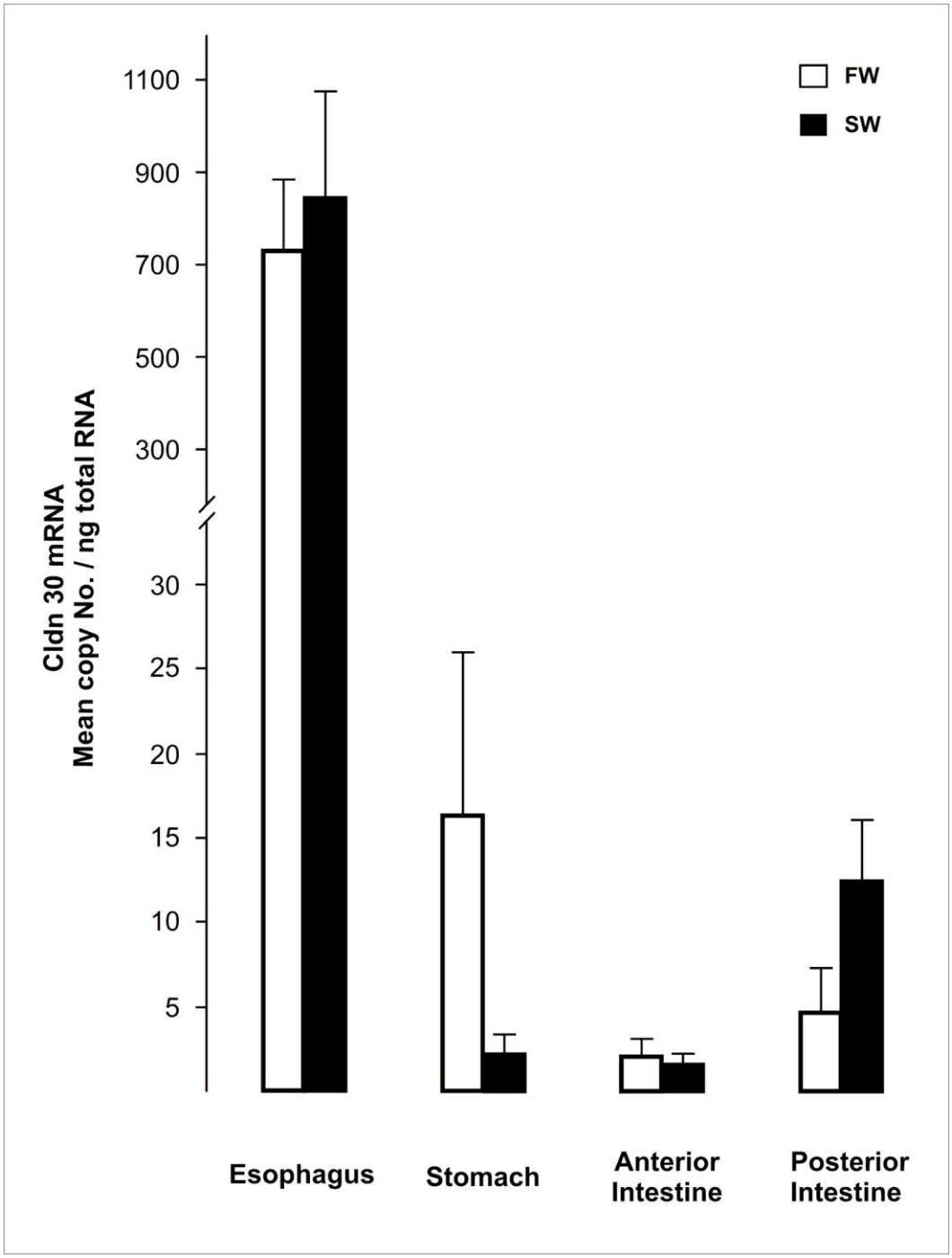


Figure 1.11: Cldn30 mRNA expression along the GI tract of SW and FW tilapia. The expression of cldn30 is the highest in the esophagus among four segments examined. There is no statistical difference in cldn30 between SW and FW fish GI tract segments. Values are mean +/- SEM, n= 6 tilapia per each treatment group.

Expression of Claudins in Response to Salinity Challenge:

The dynamics of claudin expression in the posterior intestine were investigated in response to FW and SW-challenge at 5 different time points (0, 1, 4, 7, 21 days). The first sample was taken at time 0, prior to the salinity challenge. Fish sham-transferred (FW to FW or SW to SW) were used as negative controls.

The time-course of changes in the cldn3c, cldn28a, and cldn30 mRNA expression in response to salinity challenge was examined using SYBR green real-time PCR analysis.

Transfer of SW tilapia to FW, induced an approximately 2-fold increase in cldn3c mRNA expression in the posterior intestine in 24 hours relative to the control group, ($p<0.05$; Fig. 1.12A). After 4 days, cldn3c expression dropped back to control levels where levels were maintained throughout the remainder of the 21-day challenge period. In the control group (SW to SW), the expression of cldn3c mRNA did not change significantly throughout the time course of salinity challenge (Fig. 1.12A).

Transfer of FW tilapia to SW showed a reverse pattern to that of FW-challenged fish (Fig. 1.12B). In this case, cldn3c mRNA levels decreased 6.1 folds within 24 hour after SW transfer. Then, cldn3c recovered to original levels within 4 days. In the control group (FW to FW), the expression of cldn3c mRNA did not change significantly throughout the 21 days of challenge (Fig. 1.12B).

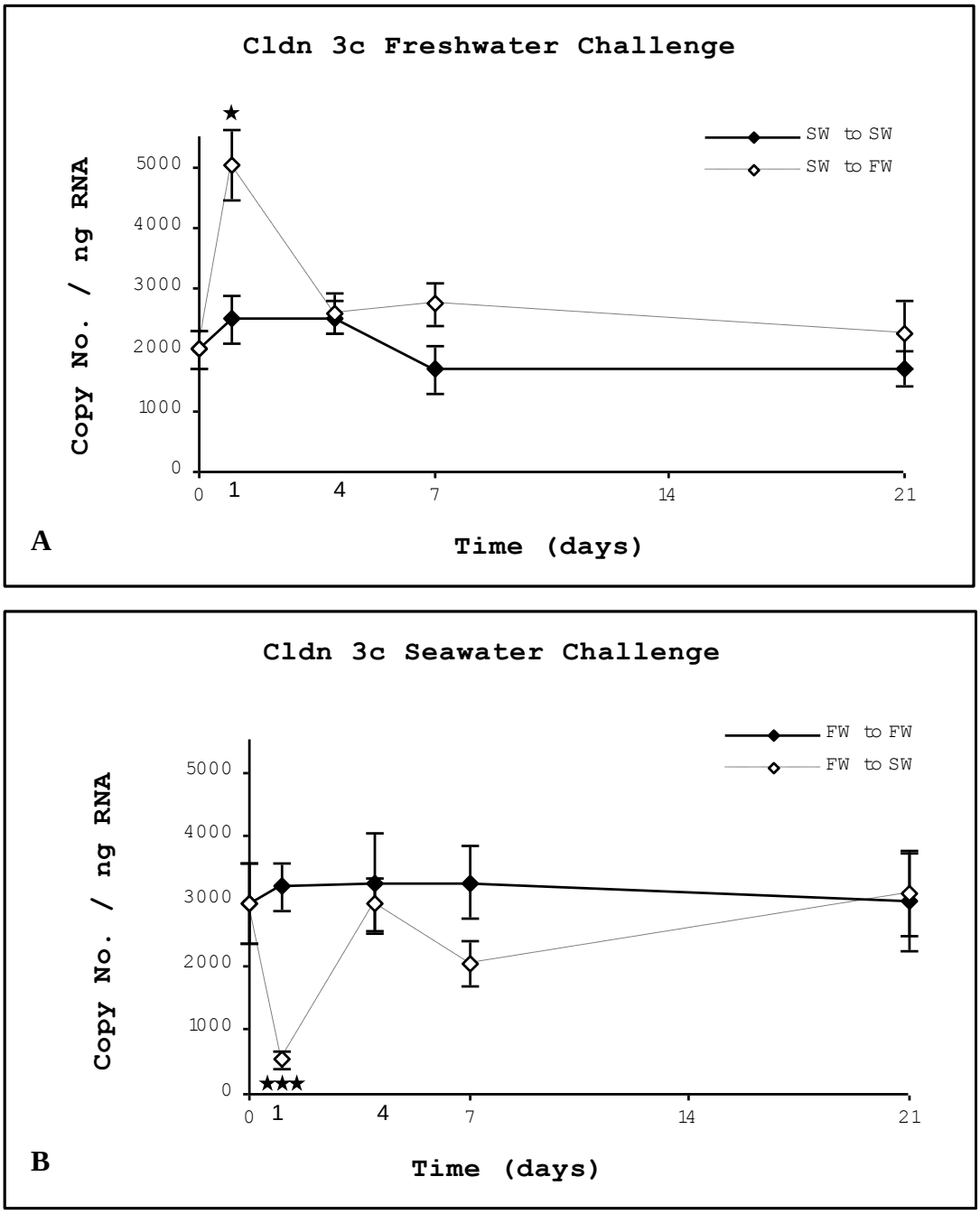


Figure 1.12: The time-course of claudin3c mRNA expression of the posterior intestine in response to FW and SW transfer. The effects of FW-challenge (A) and SW-challenge (B) over 21 days. Values are mean +/- SEM, n= 7 or 8 tilapia per each treatment group and time point. Solid lines represent negative controls (fish sham-transferred), dotted line represents the transfer of opposite salinity condition (SW to FW or FW to SW). "★" indicates statistical significance between treatment groups (★: $p < 0.05$), (★★★: $p < 0.0001$).

Transfers of neither SW tilapia to FW nor FW tilapia to SW induced significant changes in cldn28a expression relative to control fish (Fig. 1.13). In both cases, the initial expression of cldn28a was very low relative to cldn3c and cldn30 with basal levels approximating 2 copy numbers/ng RNA (Figs. 1.13A and B).

The general expression levels of cldn30 were higher (RNA copy number per ng RNA) than that of cldn28a, but lower than that of cldn3c in the tilapia posterior intestine during the 21-day salinity challenge period (Fig. 1.14). After 21 days transfer to FW, expression of cldn30 decreased relative to control (sham-transferred) (Fig. 1.14A).

Transfer of FW fish to SW caused fluctuations in cldn30 expression during 21 days of salinity challenge period. While the expression of cldn30 was 3.3 folds lower in SW challenged fish relative to control after 7 days of transfer, levels were 4.5 fold higher in SW challenged fish relative to controls after 21 days of transfer (Fig. 1.14B).

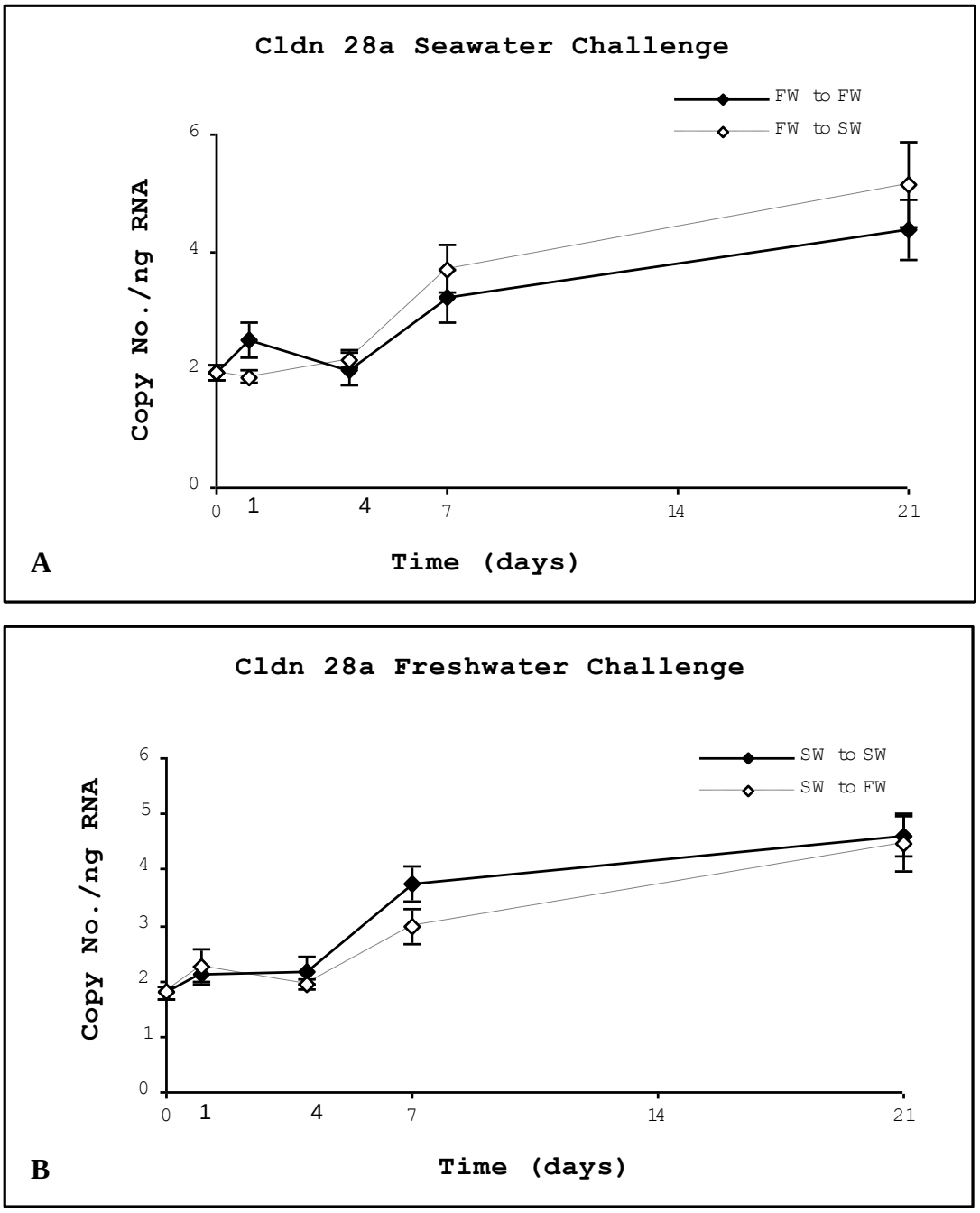


Figure 1.13: The time-course of claudin28a mRNA expression of the posterior intestine in response to SW and FW transfer. The effects of FW-challenge (A) and SW-challenge (B) over 21 days. Values are mean +/- SEM, n= 7 or 8 tilapia per each treatment group and time point. Solid lines represent negative controls (fish sham-transferred), dotted line represents the transfer of opposite salinity condition (SW to FW or FW to SW).

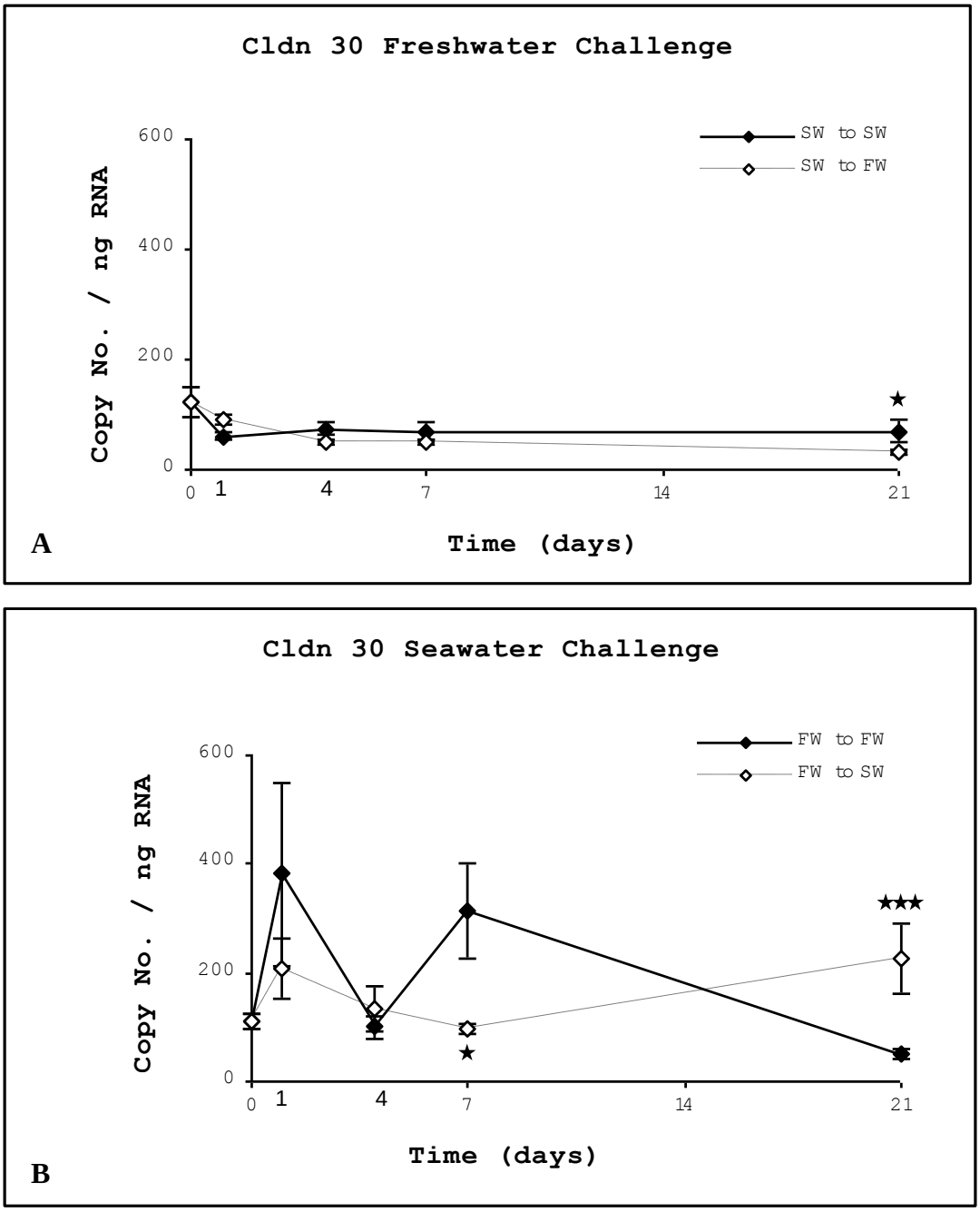


Figure 1.14: The time-course of claudin30 mRNA expression of the posterior intestine in response to SW and FW transfer. The effects of FW-challenge (A) and SW-challenge (B) over 21 days. Values are mean +/- SEM, n= 7 or 8 tilapia per each treatment group and time point. Solid lines represent negative controls (fish sham-transferred), dotted line represents the transfer of opposite salinity condition (SW to FW or FW to SW). ★ indicates statistical significance between treatment groups (★: $p < 0.05$), (★★★ **:** $p < 0.0001$).

DISCUSSION

The euryhaline teleost, Mozambique tilapia can adapt to both SW and FW. While fish face dehydration problems in SW, they must cope with severe hydration in FW. The ability to adapt two opposing salinities requires drastic changes in various osmoregulatory organs such as skin, gills, kidney and intestine (reviewed by Marshall and Grosell, 2005). Differential organization of the GI tract, gill, kidney and urinary bladder epithelia is coupled with increased ion and water uptake in SW fish than that of FW fish (Takahashi et. al., 2007; McCormick, 2001). In this project, the expression of claudin tight junction proteins in epithelia was investigated to determine their potential contributions to in regulating hydromineral balance in the GI tract of tilapia.

Like mammalian, the fish GI tract is anatomically divided into specialized segments: esophagus, stomach, and small intestine. In the cross section of each segment, from the lumen to plasma, the GI tract includes similar morphological layers: mucosa, submucosa, smooth muscle layers and an outermost connective tissue. In fish, each segment plays different roles for adapting to diverse salinities. One of the drastic differences of these segments is probably the organization of epithelia, which is located in the mucosa layer, next to the lumen. The properties of epithelia are believed to be unique in different parts of the GI tract. Tight junctions are one of the important contributors to this diversity. The expression of claudins is not only diverse in different parts of the GI tract, but may also be different within an individual segment (Rahner et al., 2001; Holmes et al., 2006). In fugu, 56 claudin isoforms have been identified, and the distribution of claudins seems to be tissue specific in different fish species (Loh et al., 2004; Tipsmark et. al., 2008c). Moreover, in

combination the claudin members could add an enormous diversity to the properties of paracellular transport, and tissue specific responsiveness. In general, claudins can be classified into two groups with respect to their cation permeability: claudins having tightening potential (mammalian claudins 1, 3, 4, 5, 11, and 14, for example), and claudins having pore forming properties (claudin 2, 7, 15, and 16, for example) in MDCK and LLC-PK1 cell lines (Amasheh et al., 2002; Wen et. al., 2004; Alexandre et al., 2005; Van Itallie et al., 2001; Van Itallie et al. 2003). However, the tightening ability of individual claudin depends on the composition and abundance of other claudins that might line the paracellular pores of different tissues (Krause et. al., 2008; Yu, 2003).

Since identity of claudin genes in fish has only recently been established in fish, there are no available antibodies against claudin proteins in fish. However, claudins are evolutionary highly conserved and present in all animal species including those invertebrates to vertebrates examined to date (Furuse and Tsukita, 2006; Behr et al., 2003; Asono et al., 2003). Since mammalian claudins show high homology to fish claudins, the specificity of various mammalian antibodies raised against claudins had been tested, and it was detected that rabbit-anti claudin 3 and mouse-anti claudin 4 antibodies showed high specificity in tilapia gill (Tipsmark et al., 2008a). In the present study, the subcellular localizations of claudin 3 and 4-like proteins in the GI tract of tilapia were reported. Throughout the GI tract, staining of these proteins was detected in all three segments (esophagus, stomach, and intestine), and localization was mainly found in the epithelia as previously reported for the mammalian GI tract.

In SW eel, the esophagus is the main part of the GI tract for the removal of salt (Hirano and Mayer-Gostan, 1976; Parmelee and Renfro, 1983; Nagashima and Ando, 1994), and act to decrease the osmolality of the ingested SW, likely essential for the intestine to absorb water. The SW eel esophagus is permeable to Na^+ and Cl^- ions, but not water (Marshall and Grosell, 2005; Hirano and Mayer-Gostan, 1976). The movement of Na^+ is driven by basolateral Na^+-K^+ ATPase. Na^+ is believed to be taken into the epithelial cells by apically located Na^+-Cl^-, Na^+-K^+-$2Cl^-$ co-transporters, Na^+-H^+ exchangers and/or Na^+ channels (Parmelee and Renfro, 1983; Marshall and Grosell, 2005). Desalination is crucial because the intestine cannot absorb water if the content of luminal salt is too high (Skadhauge 1969; Ando et al., 2003). After the absorption of salt by the esophagus, the osmolality of the plasma increases. In eel, the heart is in very close proximity to the esophagus; and accordingly, the blood with high salt content is delivered to the heart through a short circulation circuit. Likewise, the heart pumps this blood to the gills which are again in close proximity of the heart; and consequently, NaCl is efficiently excreted back into the environment by the gills using a short blood vessel circuit (Ando et al., 2003). The uptake of water is minimal or absent in the esophagus of SW eel (Hirano and Mayer-Gostan, 1976; Ando et al., 2003). In the intestine pores in paracellular pathways are large enough to allow the passage of water molecules (Angelow et al., 2006; Krause et al., 2008), and they are believed to be one of main routes for water uptake through the intestine (Marshall and Grosell, 2005). Epithelia display a range of resistance along the GI tract and tight junctions are the main determinant for the transepithelial resistance (Ma and Anderson, 2006). Esophagus is classified as having “tight” tight junctions (Ma and

Anderson, 2006). In accordance with these reports, it was expected that tight junctions should be tight enough to prevent the diffusion of luminal sea water through paracellular routes in marine fish esophagus. In addition, uptake of Na^+ which depends of the basolateral Na^+ pumps, and establishment of "tight" tight junctions could prevent the back leakage of Na^+ into the lumen by creating cation discriminating pores.

FW fish that live in a highly hypoosmotic environment relative to their plasma must minimize water uptake through their epithelia including in the GI tract. Therefore, hydrophobic tight junctions should form tight barriers in the paracellular spaces in the epithelia along the GI tract. In addition, since FW is very poor in salt concentration, the paracellular pathways are likely to be built with high amounts of cation discriminating claudins in tight junction structures throughout the GI tract.

Cldn-3 is present along the gastrointestinal tract of mammals (Rahner et al., 2001; Holmes et al., 2006; Haworth et al., 2005). Cldn-3 overexpression has been reported to reduce paracellular permeability of human airway epithelia lines (Coyne et al., 2003). Conversely, reduction in cldn-3 expression caused increased paracellular permeability in Caco-2 intestinal cell line epithelia (McLaughlin et al., 2004). In the mouse kidney, cldn-3 was detected in the ascending loop of Henle, distally convoluted tubule, and collecting duct of nephrons where tight junctions are tighter, but its expression was absent in the proximal segment where tight junctions are leakier (Kiuchi-Saishin et al., 2002). Phosphorylation of cldn 3 with PKA decreased TJ strength in ovarian cancer cell line OVCA 433, as shown by decreased TER and increased TJ permeability (D'Souza et al., 2005). In addition a mutation in cldn3 mimicked the phoshorylation state of cldn 3 and caused an increase in paracellular

flux and reduction in TER (D'Souza et al., 2005). Cldn-3 isoforms are also present in fish (*Tetraodon sp.)* kidney, skin and intestinal epithelium and proposed to contribute to form "tight" TJs (Bagherie-Lachidan et al, 2008). The majority of the reports suggest that cldn 3 has a tightening potential for TJ (Krause et al., 2008).

Similar to cldn-3, **cldn-4** is suggested to be classified as a sealing (tightening) claudin (Krause et al., 2008). Cldn-4 was shown to form pores that discriminate against cations (Van Itallie et al., 2001). When this claudin was expressed under an inducible promoter in MDCK cells, it induced a dose dependent increase in TER. Inhibition of methyl transferases increased cldn-4 expression and caused an increase in TER in a bladder tumor cell line. Knockdown of the expression of cldn-4 in this cell line dropped the TER (Boireau et al., 2007). Like cldn-3, cldn 4 is highly present in the tighter parts of the nephron (Kiuchi-Saishin et al., 2002).

The majority of reports suggest that both cldn-3 and -4 have a tightening potential for TJs. However, the tightening ability of individual claudin depends on the presence and abundance of other claudins and the expression and organizations of other types of tight junctional proteins (Krause et al., 2008; Van Itallie et al., 2008).

Consistent with these reports, high expression of both cldn-3-, and cldn-4-like proteins in the SW tilapia GI tract were detected (Figs. 1.3-1.8). The **esophagus** is the main desalting organ in marine fishes. In both SW and FW tilapia esophagus, both proteins are localized primarily in the epithelial layer, between the epithelial cells (Fig. 1.3 and 1.6). Cldn-4-like staining was mainly at the apical sides of the epithelia. Additionally, some staining was observed on the basal side of the cells (Fig. 1.6). Cldn-3-like protein was not

solely in the apicolateral regions, and staining was observed throughout the apical and basolateral plasma membranes (Fig. 1.3). In the immunostaining images, staining of both cldn-3- and especially cldn-4-like proteins seemed stronger in FW tilapia than SW tilapia esophagus. This is not surprising because, unlike the SW eel esophagus, the esophagus of FW fish is largely impermeable to ions (Hirano and Mayer-Gostan, 1976). According to immunohistochemistry images, the stronger expressions of claudin proteins in the esophagus of FW tilapia than that of SW fish is similar to that was previously reported in tilapia gill (Tipsmark et al., 2008a). Both these proteins could form hydrophobic barriers, probably by decreasing the size or number of pores, and/or by creating cation discriminating pores in the paracellular spaces. This would result in the establishment of "tight" TJs in the esophageal epithelia.

Unlike mammalian tissues which express only one cldn-3 protein, in fish, four cldn-3 isoforms have been identified. Like cldn-3, multiple cldn-4-like isoforms are believed to be present in fish (Loh et al.). In Japanese puffer fish (*Takifugu rubribes*) 17 genes are believed to be related to mammalian cldn-3 and -4 (Loh et al., 2004). In this project, claudin antibodies raised in mammals. Therefore, it is possible, one or more cldn-3 or cldn-4-like isoforms were detected in the GI tract. The precise teleost isoforms remain to be identified.

In addition to expression of cldn-3 and -4 like proteins, the **morphological differences** in the tilapia GI tract between SW and FW-acclimated was examined. In the SW eel **esophagus**, a relatively higher numbers of mucosal folding were reported, likely needed to increase surface area (Yamamoto and Hirano, 1978). Similarly, more folding of the esophageal mucosa in SW compared with FW-acclimate fish was observed (Fig. 1.3A

and A'). SW-acclimated European and Japanese eels have columnar epithelia which line the mucosal layer of the esophagus, which differs from stratified epithelium of FW adapted fish (Hirano and Mayer-Gostan, 1976; Yamamoto and Hirano, 1978). In this study, it was observed that SW-acclimated tilapia esophagus has columnar epithelia, but FW tilapia seems to have epithelia in the transition state from columnar to stratified type (Figs 1.3B and B'). In SW tilapia, higher vascularization in the submucosa was detected which is consistent with previous reports (Fig. 1.3A and A') (Yamamoto and Hirano, 1978). Tight junctions are dynamic structures (Van Itallie et al., 2004). In response to abrupt environmental salinity changes, they can change their expression (gene level) and organizations (protein level) quickly (explained further below for salinity challenge experiment part) (Tipsmark et al 2008a). In addition to changes in the structure of tight junctions, the number and activity of various transporters such as various Na^+ co-transporters and Na^+ pumps have been reported to change within 4 days of salinity transfer (Tipsmark et al., 2008b). And ultimately, after long term adaptation, the whole epithelial structure changes at tissue level. For example, in FW adapted eel, the number of esophageal goblet cells is higher than in SW-acclimated fish. Mucus cells secrete hydrophobic mucus. This mucus could cover the surface of the epithelial layer and prevent water diffusion through the epithelial cell along the GI tract of FW-acclimated fish.

In SW eel **stomach**, some Na^+, Cl^-, and water permeability is detected (Hirano and Mayer-Gostan, 1976). After the desalination by the esophagus, the stomach further dilutes the ingested water via uptake of ions, and osmolality of the luminal fluid approaches that to the plasma (Marshall and Grosell, 2005; Ando and Nagashima, 1996; Hirano and Mayer-

Gostan, 1976). No difference for permeability of ions and water between FW and SW-acclimated eel stomach was found (Hirano and Mayer-Gostan, 1976). Because the presence of some ion and water permeability has been previously reported in fish stomach, it was not surprising to detect cldn-like proteins in the fish stomach. In addition, both cldn-3 and -4 expression was previously reported in rat stomach (Rahner et al., 2001). In the present study, it was observed that both cldn 3-like and high amounts cldn 4-like proteins in both SW and FW tilapia stomach (Figs. 1.4 and 1.7). This raises the possibility that tight junctions in the stomach might also play important roles for hydromineral balance in tilapia. Cldn 3-like immunostaining was strictly localized in the tight junctional regions in both SW and FW fish stomach (Fig. 1.4). Additionally, cldn 3-like staining was seen in the apical sides of stomach epithelial cells in FW fish, this might be a reflection of strong tight junctional staining at the apico-lateral sides. Strong cldn 4-like staining was present in both apico-lateral and basal sides of gastic pits in SW and FW fish stomach (Fig. 1.7). Both the cldn 3 and cldn 4-like protein staining seemed stronger in FW than in SW stomach epithelia. These proteins might form barriers in the paracellular pathways to restrict diffusion of water, and Na^+ discriminating pores to prevent the backflow of Na^+, which could be more important for hydromineral balance in FW environments. In addition, cldn-4-like proteins were observed between the tubular glands consisting of digestive enzyme secreting chief cells and hydrochloric acid secreting parietal cells beneath the gastric pits. It might be especially important for fish to maintain stable barriers between the tubular gland cells during the appropriate delivery of digestive enzymes and hydrochloric acid from the deeper zones of the mucosa to the luminal side. High cldn 4-like protein expression

between various types of cells in the mucosa layer of stomach might be important to maintain a stable bi-directional movement of various molecules. In rat stomach, cldn-3 and -4 were both expressed in the surface epithelium and in gastric glands. Cldn-4 expression was particularly strong in the gastric glands just as the observations in tilapia stomach (Rahner et al., 2001).

The intestine part of the GI tract plays crucial roles for osmoregulation of marine fishes (Marshall and Grosell, 2005). After desalination of ingested SW mainly by the esophagus, and to a lesser degree in the stomach, the fluid reaches the intestine (Hirano and Mayer-Gostan, 1976, Parmelee and Renfro, 1983). Absorption of water requires obligatory Na+ and Cl^- absorption, but the exact mechanism of water intake from the intestine is not known (Grosell and Genz, 2006; Lorentz, 1995; Marshall and Grosell, 2005). Uptake of Na^+ and Cl^- into the epithelia is mediated by apically located Na^+-Cl^-, Na^+-K^+-$2Cl^-$ co-transporters and HCO_3^--Cl^- anion exchangers (Frizzell et al., 1979; Halm et al., 1985) and the energy for transport is provided by basolateral Na^+-K^+ ATPases (Lorentz, 1995). Lumenal water follows the salt using both transcellular and paracellular pathways (Marshall and Grosell, 2005; Ando et al., 2003; Masyuk et al., 2002). In addition, various co-transporters such as Na^+-K^+-$2Cl^-$, K^+-Cl-, and Na^+-glucose transporters have been proposed to pump water into the cells through transcellular pathways in marine teleost intestine (Loo et al., 1999; Zeuthen and MacAulay, 2002). Excessive salt in the plasma is actively extruded by the gills (Marshall and Grosell, 2005).

Even though tight junctions are classified as “leaky” in the mammalian intestine, FW fish must cope with hydration problems by mainly reducing water permeability and

increasing uptake of various ions from the dilute environment. Therefore, in the FW fish intestine, claudins must form "tight" barriers between cells and/or they prevent leakage of Na^+ via paracellular pathways by forming cation discriminating pores in TJs. Although previous in vitro studies have reported that FW anterior and posterior intestines have higher or similar water permeability as SW intestines (Mainoya, 1982; Rawdon and Cornish, 1973), FW fish do not drink water and the luminal water content is much smaller and limited to the water content of the feed. Excessive water intake is eliminated by the kidney. On the other hand, the intestinal epithelia of SW-adapted fish display high water and ion permeability (McCormick, 2001) because water absorption depends on salt absorption. Therefore, in SW fish intestine, tight junctions should allow the movement of water through the paracellular pathways if these routes play roles in water absorption as widely believed. In our immunohistochemistry studies, cldn-3-like protein was highly expressed in the tight junctional areas, and the intensity of cldn 3-like staining was similar between SW and FW-adapted tilapia. Cldn-4 staining was present at the apical and basal sides of the epithelia. Basal side cldn 4-like staining was obvious on the lower halves of SW intestinal villi. This might indicate additional roles of cldn 4-like proteins on the basal side of the epithelia such as in adhesion, or in contributing to additional permeability properties of SW fish intestinal epithelia. Additionally, they could form a protein pool on the basal side of the cells to modify TJ structure immediately. The intensity of apical cldn 4-like staining seemed higher in FW fish intestine, but this might artificially stem from the orientation of the cryosection during preparation.

Cldn3c, 28a, and 30 show approximately 85% similarity to mammalian cldn-3 and -4 (Tipsmark et al., 2008c). Here the mRNA expression patterns of these three genes in SW and FW fish along the GI tract, and in response to salinity transfer in the posterior intestine were examined. The expression levels of these genes were highest in the esophagus among the four different segments of the tilapia gut in both FW and SW fish. This indicates tissue specific expression along the GI tract, and their potential importance in epithelial permeability during esophageal osmoregulation.

The expression patterns of four Tncldn3 genes have been previously investigated in two-week acclimated SW and FW Tetraodon intestines. The expression level of Tncldn3c was the lowest relative to other three isoforms in the intestine, and levels did not differ in fish from different salinities. On the other hand, Tncldn3a, Tncldn3b and Tncldn3d genes were significantly reduced upon SW acclimation (Bagherie-Lachidan et al., 2008). The findings on cldn3c expression in one-month SW and FW acclimated tilapia is consistent with the report on Tetraodon intestine. Cldn3c expression in the anterior intestine and posterior intestine did not differ between SW and FW-acclimated tilapia. However, in the salinity challenge experiments, cldn3c expression was significantly reduced and increased within 24 hours following SW and FW transfer, respectively (Fig 1.12). Four days after the transfer, the levels of cldn3c returned back to their original levels. This indicates that cldn3c is rapidly regulated in response to environmental salinity. In a specific tissue, certain claudin proteins, which are expressed widely, might form a "housekeeping protein pool" in TJs to provide steady permeability properties for the epithelia in normal conditions. In response to an alteration in the environment, changes in the expression and/or organization

of a specific set of claudins might play "fine-tuning" roles to give certain permeability qualities a specific tissue (Van Itallie et al., 2006). Cldn3c might play a quick "fine-tuning" role to adjust paracellular permeability in altered environmental salinity conditions, whereby it favors adaptation to FW environments. The expression levels of cldn28a did not change significantly in the posterior intestine during salinity transfer. The expression of cldn30 was lowered in FW challenged fish, and induced in SW challenged fish after 21 days from the transfer. The fluctuation in cldn30 expression in "FW to FW" and "FW to SW" transferred fish at 7 days following the transfer might stem from factors other than salinity such as stress. Cldn30 expression differed from cldn3c in two ways: firstly, contrary to cldn3c expression, which displays a quick response upon salinity transfer, cldn30 responded to salinity in a long-term fashion. Secondly, unlike cldn3c, which might have a sealing role in FW fish posterior intestine as proposed in various tissues in Tetraodon (Bagherie-Lachidan et al., 2008), cldn30 might have a different function from cldn3c such as a pore forming role in the paracellular spaces of SW fish posterior intestine.

SUMMARY

During salinity acclimation, changes in the properties of epithelia of GI tract are essential for osmotic homeostasis. Various claudins were detected in different segments of the fish GI tract. The subcellular localizations of cldn 3-like and 4-like proteins were reported in esophagus, stomach, and intestine of GI tract.

Claudin proteins are highly dynamic structures (Van Itallie et al., 2004; Matsuda et al., 2004), and in response to exogenous stimuli, can change their expression and

reorganization quickly. In the current study, little difference in claudin expression between SW and FW fish was found. However, certain claudins may respond quickly to salinity transfer and provide a "fine-tune" mechanism for epithelial permeability. This appears to be the case for cldn3c whose posterior intestine mRNA levels rapidly increased upon FW and declined upon SW exposure. Along with the alteration of tight junctional organization, the number and activity of the various channels, transporters, and pumps on transcellular pathways may also change (Evans et al., 2005; Marshall, 2002). In addition to short term responses, the epithelia may also reorganize at the tissue level to maintain osmotic homeostasis over the long-term. Two main morphological changes were observed in our studies: an alteration of epithelial cells such as goblet cells which form a hydrophobic mucus layer on the surface of epithelial cells, and second, the reorganization of apical sides of the epithelial cell membranes by decreasing or increasing the apical surface area. These morphological changes were previously reported between SW and FW-acclimated fish esophagus, and can be expected throughout the GI tract.

CONCLUSIONS

Claudin proteins are widely believed to be one of main structural and functional components of TJs. The importance of these proteins has been shown in various diseases including mutations in claudins, and with knockout / knockdown experiments in mammals. However, very little is known about the individual functions of these transmembrane proteins, their regulations and how they might participate in forming barriers or change the size or number of pores within paracellular spaces. In this study, the subcellular

localizations of claudin-like proteins in the tilapia gastrointestinal tract are mainly localized in the epithelia as previously reported in mammals. Secondly, the expression levels of certain claudins change dynamically at the mRNA level in response to changes in environmental salinity. This might indicate differential expression and TJ contributions of claudins to the maintenance of hydromineral balance, likely via changing the permeability of the GI tract epithelia of tilapia.

FUTURE DIRECTIONS

Determining the expression profiles of claudin proteins in the osmoregulatory organs including the esophagus and intestine is a crucial step to uncover the roles of these proteins for hydromineral balance in fish. Therefore, all fish claudins should be identified, and fish specific antibodies must be produced for immunohistochemistry analyses. In addition, protein expression analyses should be complemented by mRNA expression analyses. The localization of various claudin mRNA can be detected by *in situ* hybridization. In our lab, it has been already prepared and tested the specificity of cldn3c, cldn28a, and cldn30 probes, and completed the preparation of the various frozen tissue sections for in situ hybridization.

The mechanism of regulation of tight junction permeability in fish is largely unknown. The involvement of hormones, such as cortisol and prolactin in altering epithelial permeability for Na^+ in response to salinity challenge has been previously reported (Hirano, 1980). Likewise, the importance of these hormones along with some others has been shown during SW and FW adaptations. While cortisol is a crucial hormone for the SW adaptation,

prolactin plays a key role for FW adaptation. The effects of cortisol and prolactin on claudin expression are warranted considering their importance to osmoregulation in fish. Studies in mammals report that glucocorticoids and prolactin regulate of claudin expression. Hormonal regulation of claudin expression can be tested by altering the concentration of these hormones in fish in certain conditions and measuring the levels of claudins using real-time PCR and microarrays analyses. Additionally, expression of other key signaling molecules that may induce or suppress claudin expression could be investigated in the future.

REFERENCES

Alexandre MD, Lu Q, Chen YH. 2005. Overexpression of claudin-7 decreases the paracellular Cl− conductance and increases the paracellular Na+ conductance in LLC-PK1 cells. J. Cell Sci. 118:2683–93

Amasheh S, Meiri N, Gitter AH, Schoneberg T, Mankertz J, Schulzke JD, Fromm M. 2002. Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells. J Cell Sci. 115:4969–4976.

Anderson JM, Van Itallie CM. 2008. Tight junctions. Curr Biol. 18(20):R941-943.

Ando M and Nagashima K. 1996. Intestinal Na + and Cl– levels control drinking behavior in the seawateradapted eel Anguilla japonica, J.Exp.Biol., 199, 711–716.

Ando M, Mukuda T, Kozaka T. 2003. Water metabolism in the eel acclimated to sea water: from mouth to intestine. Comp Biochem Physiol B Biochem Mol Biol. 136(4):621-633.

Angelow S, Kim KJ, Yu ASL. 2006. Claudin-8 modulates paracellular permeability to acidic and basic ions inMDCKII cells, J. Physiol.-London. 571. 15–26.

Asano A, Asano K, Sasaki H, Furuse M, Tsukita S. 2003. Claudins in Caenorhabditis elegans: their distribution and barrier function in the epithelium. Curr Biol. 13(12):1042-1046.

Bagherie-Lachidan M, Wright SI, Kelly SP. 2008. Claudin-3 tight junction proteins in Tetraodon nigroviridis: cloning, tissue-specific expression, and a role in hydromineral balance. Am J Physiol Regul Integr Comp Physiol. 294(5):R1638-1647.

Behr M, Riedel D, Schuh R. 2003. The claudin-like megatrachea is essential in septate junctions for the epithelial barrier function in Drosophila. Dev Cell. 5(4):611-620.

Beyenbach KW. 2004. Kidneys sans glomeruli. Am J Physiol Renal Physiol. 286(5): F811–827.

Boireau S, Buchert M, Samuel MS, Pannequin J, Ryan JL, Choquet A, Chapuis H, Rebillard X, Avances C, Ernst M, Joubert D, Mottet N, Hollande F. 2007. DNA-methylation-dependent alterations of claudin-4 expression in human bladder carcinoma, Carcinogenesis. 28, 246–258.

Boutet I, Long Ky CL, Bonhomme F. 2006. A transcriptomic approach of salinity response in the euryhaline teleost, Dicentrarchus labrax. Gene. 379:40-50.

Brizuela BJ, Wessely O, DeRobertis EM. 2001. Overexpression of the Xenopus tight-junction protein claudin causes randomization of the left-right body axis. Dev Biol., 230(2):217-229.

Bustin SA. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 25:169–193.

Christoffels A, Koh EGL, Chia J, Brenner S, Aparicio S, Venkatesh B. 2004. Fugu genome analysis provides evidence for a whole-genome duplication early during the evolution of ray-finned fishes. Mol Biol Evol 21: 1146–1151.

Colegio OR, Van Itallie CM, McCrea HJ, Rahner C, Anderson JM. 2002. Claudins create charge-selective channels in the paracellular pathway between epithelial cells. Am J Physiol Cell Physiol. 283(1):C142-147.

Colegio OR, Van Itallie CM, McCrea HJ, Rahner C, Anderson JM. 2002. Claudins create charge-selective channels in the paracellular pathway between epithelial cells. Am J Physiol Cell Physiol. 283(1):C142-147.

Coyne CB, Gambling TM, Boucher RC, Carson JL, Johnson LG. 2003. Role of claudin interactions in airway tight junctional permeability. Am J Physiol Lung Cell Mol Physiol. 285(5):L1166-1178.

D'Souza T, Agarwal R, Morin PJ. 2005. Phosphorylation of claudin-3 at threonine 192 by cAMP-dependent protein kinase regulates tight junction barrier function in ovarian cancer cells. J Biol Chem. 280(28):26233-26240.

Evans DH, Piermarini PM, Choe KP. 2005. The multifunctional fish gill: Dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiol Rev 85: 97–177.

Fiess JC, Kunkel-Patterson A, Mathias L, Riley LG, Yancey PH, Hirano T, Grau EG. 2007. Effects of environmental salinity and temperature on osmoregulatory ability, organic osmolytes, and plasma hormone profiles in the Mozambique tilapia (Oreochromis mossambicus). Comp Biochem Physiol A Mol Integr Physiol. 146(2):252-264.

Frizzell RA, Smith PL, Vosburgh E, Field M. 1979. Coupled sodium-chloride influx across brush border of flounder intestine. J Membr Biol. 46(1):27-39.

Furuse M, Fujimoto K, Sato N, Hirase T, Tsukita S, Tsukita S. 1996. Overexpression of occludin, a tight junction-associated integral membrane protein, induces the formation of intracellular multilamellar bodies bearing tight junction-like structures. J Cell Sci. 109 (Pt 2):429-35.

Furuse M, Sasaki H, Fujimoto K, Tsukita S. 1998. A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts. J Cell Biol. 143(2):391-401.

Furuse M, Tsukita S. 2006. Claudins in occluding junctions of humans and flies. Trends Cell Biol. 16(4):181-188.

Gonzalez-Mariscal L, Betanzos A, Nava P, Jaramillo BE. 2003. Tight junction proteins. Prog. Biophys. Mol. Biol. 81, 1-44.

Grosell M, Genz J. 2006. Ouabain-sensitive bicarbonate secretion and acid absorption by the marine teleost fish intestine play a role in osmoregulation. Am J Physiol Regul Integr Comp Physiol. 291(4):R1145-1156.

Grosell M, Wood CM, Wilson RW, Bury NR, Hogstrand C, Rankin C, Jensen FB. 2005. Bicarbonate secretion plays a role in chloride and water absorption of the European flounder intestine. Am J Physiol Regul Integr Comp Physiol. 288(4):R936-946.

Halm DR, Krasny EJ, and Frizzell RA. 1985. Electrophysiology of flounder intestinal mucosa. II. Relation of the electrical potential profile to coupled NaCl absorption. J Gen Physiol 85: 865–883.

Halm, D. R., Krasny, E. J. and Frizzell, R. A. 1985. Electrophysiology of flounder intestinal mucosa.II. Relation of the electrical potential profile to coupled NaCl absorption., J.Gen.Physiol., 85, 865–883.

Haworth KE, El-Hanfy A, Prayag S, Healy C, Dietrich S, Sharpe P. 2005. Expression of Claudin-3 during chick development. Gene Expr Patterns. 6(1):40-44.

Hirano, T., Mayer-Gostan, N., 1976. Eel esophagus as an osmoregulatory organ. Proc. Natl. Acad. Sci. USA 73, 1348–1350.

Hiroi J, Kaneko T, Uchida K, Hasegawa S, Tanaka M. 1998. Immunolocalization of Vacuolar-Type H(+)-ATPase in the Yolk-Sac Membrane of Tilapia (Oreochromis mossambicus) Larvae. Zoolog Sci. 15(4):447-453.

Holmes JL, Van Itallie CM, Rasmussen JE, Anderson JM. 2006. Claudin profiling in the mouse during postnatal intestinal development and along the gastrointestinal tract reveals complex expression patterns. Gene Expr Patterns, 6(6):581-588.

Isaia J, Hirano T. 1976. Effect of environmental salinity change on osmotic permeability of the isolated gill of the eel, Anguilla anguilla L. J Physiol (Paris). 70(6):737-47.

Kelly SP, Wood CM. 2001. Effect of cortisol on the physiology of cultured pavement cell epithelia from freshwater trout gills. Am J Physiol Regul Integr Comp Physiol 281: R811–R820.

Kiuchi-Saishin Y, Gotoh S, Furuse M, Takasuga A, Tano Y, Tsukita S. 2002. Differential expression patterns of claudins, tight junction membrane proteins, in mouse nephron segments. J Am Soc Nephrol. 13(4):875-86.

Krause G, Winkler L, Mueller SL, Haseloff RF, Piontek J, Blasig IE. 2008. Structure and function of claudins. Biochim Biophys Acta. 1778(3):631-645.

Laurent P, Kirsch R, Fontaine MM. 1975. Structural modifications of the esophagus linked to osmoregulation in eels. C R Acad Sci Hebd Seances Acad Sci D. 280(19):2227-2229.

Lignot JH, Cutler CP, Hazon N and Cramb G. 2002. Immunolocalisation of aquaporin 3 in the gill and the gastrointestinal tract of the European eel Anguilla anguilla (L.), J.Exp.Biol., 205, 2653–2663.

Loh YH, Christoffels A, Brenner S, Hunziker W, Venkatesh B. 2004. Extensive expansion of the claudin gene family in the teleost fish, Fugu rubripes. Genome Res 14: 1248–1257.

Loo DD, Hirayama BA, Meinild AK, Chandy G, Zeuthen T, Wright EM. 1999. Passive water and ion transport by cotransporter. J. Physiol. 518, 195–202.

Loretz CA and Fourtner CR. 1988. Functional characterization of a voltage-gated anion channel from teleost fish intestinal epithelium, J.Exp. Biol., 136, 383–403.

Loretz CA. 1995. Electrophysiology of ion transport in teleost intestinal cells. In: Fish Physiology, Cellular and Molecular Approaches to Fish Ionic Regulation, vol. 14, edited by Wood CM, Shuttleworth TJ. San Diego, CA: Academic.

Ma and Anderson, 2006. Tight junctions and intestinal barrier. In: Johnson LR, editor. Physiology of the gastrointestinal tract. Volume 2, New York: Raven Press. p. 1559-1594.

Madsen SS, Jensen LN, Tipsmark CK, Kiilerich P, Borski RJ. 2007. Differential regulation of cystic fibrosis transmembrane conductance regulator and Na+,K+ -ATPase in gills of striped bass, Morone saxatilis: effect of salinity and hormones. J Endocrinol. 192(1):249-260.

Mainoya JR. 1982. Water and NaCl absorption by the intestine of the tilapia Sarotherodon mossambicus adapted to fresh water or seawater and the possible role of prolactin and cortisol. J Comp. Physiol. 146:1-7

Marshall WS and Grosell M. 2005. Ion transport, osmoregulation and acidbase balance. *Physiology of Fishes* (3rd ed.) edited by Evans D and Claiborne JB. Boca Raton, FL: CRC. P: 177-198.

Marshall WS. 2002. Na^+, Cl^-, Ca^{2+} and Zn^{2+} transport by fish gills: Retrospective review and prospective synthesis. J Exp Zool 293: 264–283.

Marshall, W. S., Bryson, S. E., Darling, P., Whitten, M., Wilkie, M. P., Wood, C. M. and Buckland-Nicks, J. 1997. NaCl transport and ultrastructure of opercular epithelium from a freshwater adapted euryhaline teleost, Fundulus heteroclitus., J.Exp.Zool., 277, 23–37.

Masyuk AI, Marinelli RA, LaRusso NF. 2002. Water transport by epithelia of the digestive tract. Gastroenterology. 122(2):545-562.

Matsuda M, Kubo A, Furuse M, Tsukita S. 2004. A peculiar internalization of claudins, tight junction-specific adhesion molecules, during the intercellular movement of epithelial cells. J Cell Sci. 117(Pt 7):1247-1257.

McCormick SD. 2001. Endocrine control of osmoregulation in teleost fish. Am. Zool. 41, 781–794.

McDonald MD, Grosell M. (2006). Maintaining osmotic balance with an aglomerular kidney. Comp Biochem Physiol A Mol Integr Physiol. 143(4):447-458.

McLaughlin J, Padfield PJ, Burt JP, O'Neill CA. 2004. Ochratoxin A increases permeability through tight junctions by removal of specific claudin isoforms. Am J Physiol Cell Physiol. 287(5):C1412-1417.

Mozambique tilapia (Oreochromis mossambicus) in fresh water and in seawater. Gen Comp Endocrinol. 152(2-3):326-331.

Musch MW, Orellana SA, Kimberg LS, Field M, Halm DR, Krasny EJ, and Frizzell RA (1982). Na+-K+-2Cl– co-transport in the intestine of a marine teleost. Nature 300: 351–353.

Nishimura H, Imai M. Control of renal function in freshwater and marine teleosts. 1982. Fed Proc. 41(8):2355-2360.

Oliveira SS, Morgado-Díaz JA. 2007. Claudins: multifunctional players in epithelial tight junctions and their role in cancer. Cell Mol Life Sci. 64(1):17-28.

Parmelee JT and Renfro JL. (1983). Esophageal desalination of seawater in flounder: role of active sodium transport. Am J Physiol Regul Integr Comp Physiol 245: R888–R893.

Picha ME, Silverstein JT, Borski RJ. 2006. Discordant regulation of hepatic IGF-I mRNA and circulating IGF-I during compensatory growth in a teleost, the hybrid striped bass (Morone chrysops _ M. saxatilis). Gen Comp Endocrinol 147:196 –205.

Rahner C, Mitic LL, Anderson JM. 2001. Heterogeneity in expression and subcellular localization of claudins 2, 3, 4, and 5 in the rat liver, pancreas, and gut. Gastroenterology, 120(2):411-422.

Rawdon BB, Cornish EM. 1973. Intestinal water absorption in tilapia mossambica. I. Water movement in everted sacs of intestine bathed on both surfaces by identical ringer solution. Comp Biochem Physiol A Comp Physiol. 45(2):549-553.

Sakamoto T, Oda A, Narita K, Takahashi H, Oda T, Fujiwara J, Godo W. Prolactin. 2005 fishy tales of its primary regulator and function. Ann N Y Acad Sci. 1040:184-188.

Sandra O, Le Rouzic P, Cauty C, Edery M, Prunet P. 2000. Expression of the prolactin receptor (tiPRL-R) gene in tilapia Oreochromis niloticus: tissue distribution and cellular localization in osmoregulatory organs. J Mol Endocrinol. 24(2):215-224.

Sardet C, Pisam M, Maetz J. 1979. The surface epithelium of teleostean fish gills. Cellular and junctional adaptations of the chloride cell in relation to salt adaptation. J Cell Biol 80: 96–117.

Skadhauge E. 1969. The mechanism of salt and water absorption in the intestine of the eel (Anguilla anguilla) adapted to waters of various salinities. J Physiol. 204(1):135-158.

Smith NF, Talbot C and Eddy FB. 1989. Dietary salt intake and its relevance to ionic regulation in freshwater salmonids, J.Fish.Biol., 35, 749–753.

Takahashi H, Prunet P, Kitahashi T, Kajimura S, Hirano T, Grau EG, Sakamoto T. 2007. Prolactin receptor and proliferating/apoptotic cells in esophagus of the

Takahashi H, Sakamoto T, Hyodo S, Shepherd BS, Kaneko T, Grau EG. 2006. Expressions of glucocorticoid receptor in the intestine of a euryhaline teleost, the Mozambique tilapia (Oreochromis mossambicus): effect of seawater exposure and cortisol treatment. Life Sci. 78, 2329–2335

Taylor JR, Grosell M. 2006. Feeding and osmoregulation: dual function of the marine teleost intestine. J Exp Biol 209:2939–2951.

Taylor JR, Whittamore JM, Wilson RW, Grosell M. 2007. Postprandial acid-base balance and ion regulation in freshwater and seawater-acclimated European flounder, Platichthys flesus. J Comp Physiol [B]. 177(6):597-608.

Tipsmark CK, Baltzegar DA, Ozden O, Grubb BJ, Borski RJ. 2008a. Salinity regulates claudin mRNA and protein expression in the teleost gill. Am J Physiol. 294(3):R1004-1014.

Tipsmark CK, Kiilerich P, Nilsen TO, Ebbesson LO, Stefansson SO, Madsen SS. 2008c. Branchial expression patterns of claudin isoforms in Atlantic salmon during seawater acclimation and smoltification. Am J Physiol. 294(5):R1563-1574.

Tipsmark CK, Luckenbach JA, Madsen SS, Kiilerich P, Borski RJ. 2008b. Osmoregulation and expression of ion transport proteins and putative claudins in the gill of southern flounder (Paralichthys lethostigma). Comp Biochem Physiol. 150(3):265-273.

Van Itallie C, Rahner C, Anderson JM. 2001. Regulated expression of claudin-4 decreases paracellular conductance through a selective decrease in sodium permeability. J Clin Invest. 107(10):1319-1327.

Van Itallie CM, Anderson JM. 2006. Claudins and epithelial paracellular transport. Annu Rev Physiol 68: 403–429.

Van Itallie CM, Colegio OR, Anderson JM. 2004. The cytoplasmic tails of claudins can influence tight junction barrier properties through effects on protein stability. J Membr Biol. 199(1):29-38.

Van Itallie CM, Fanning AS, Anderson JM. 2003. Reversal of charge selectivity in cation or anion-selective epithelial lines by expression of different claudins. Am J Physiol Renal Physiol. 285(6):F1078-1084.

Van Itallie CM, Holmes J, Bridges A, Gookin JL, Coccaro MR, Proctor W, Colegio OR, Anderson JM. 2008. The density of small tight junction pores varies among cell types and is increased by expression of claudin-2. J Cell Sci. 121(Pt 3):298-305.

Wen H, Watry DD, Marcondes MCG, Fox HS. 2004. Selective decrease in paracellular conductance of tight junctions: role of the first extracellular domain of claudin-5, Mol. Biol. Cell 24, 8408–8417.

Wilson RW, Wilson JM, and Grosell M. 2002. Intestinal bicarbonate secretion by marine teleost fish — why and how?, Biochim.Biophys.Acta, 1566, 182–193.

Wood CM, Kelly SP, Zhou B, Fletcher M, O'Donnell M, Eletti B, Part P. 2002. Cultured gill epithelia as models for the freshwater fish gill. Biochim Biophys Acta 1566: 72–83.

Yamamoto M, Hirano, T. 1978. Morphological changes in the esophageal epithelium of the eel, *Anguilla japonica*, during adaptation to seawater. Cell. Tissue Res. 192, 25–38.

Yu AS. 2003. Claudins and epithelial paracellular transport: the end of the beginning. Curr Opin Nephrol Hypertens. 12(5):503-509.

Zeuthen T, MacAulay N. 2002. Cotransporters as molecular water pump. Int. Rev. Cytol. 215, 259–284.

C h a p t e r 2 Claudin mRNA and Protein Expressions in the Chick Intestine during the last week of Embryonic Development

ABSTRACT

Claudin protein family is the main component of tight junctions and is present in both fully developed and embryonic tissues in mammalians and birds. In adult tissues, the main role of claudins is to regulate the paracellular transport and maintain the asymmetry of the epithelial cell membranes. The presence of some of these proteins in early and late stages of chick embryonic development suggests that expressions of claudin family members are also important during development of numerous epithelial layers. In this study, the expression levels of claudin-1, -2, -3, -5, and -16, an adapter protein: ZO-2, and TGFβ3, an important signaling molecule, were investigated in the embryonic chick intestine during the last week of development using immunohistochemistry and real-time PCR analyses.

INTRODUCTION

The epithelial layer that covers the villus surface separates the lumen from the blood supply. Paracellular permeability between these two environments differs in a tissue specific manner in the epithelia of different organs and is regulated by tight junctions (TJs) (Van Itallie and Anderson, 2006; and 2004; Gonzalez-Mariscal et al., 2003; Schneeberger and Lynch, 1992). Members of the claudin protein family form the main component of TJ

strands and each family member laterally interacts with another claudin protein located in the adjacent cell in a homotypic or heterotypic manner (Krause et al., 2008; Furuse and Tsukita, 2006; Furuse et al., 1999; Furuse et al., 1996). In addition to contributing to the barrier and polarization functions of TJs in mature tissues, claudins might also be required during development in the initial establishment of epithelial sheets and the regulation of cellular signaling (Simard et al, 2006; Haworth et al., 2005; Turksen and Troy, 2001; Brizuela et al., 2001; Sonoda *et al*., 1999). Claudins 1-5, 7-13, 15, 17, and 18 have been identified in the intestinal epithelium of adult rats and/or mice (Holmes et al., 2006; Fujita et al., 2006; Inai et al., 2005; Rahner et al., 2001; Morita et al., 1999). Claudin-3 and -5 protein and transcript expression have been reported in the rat and mouse intestine (Rahner et al., 2001; Holmes et al., 2006).

The roles and locations of specific claudin protein family members during embryogenesis are not well understood. The expression patterns of claudin-1 and -3 in young chick embryos have been determined (Simard et al., 2006 and 2005; Haworth et al., 2005). The importance of the claudin-1 protein for the correct left-right patterning of the heart looping in the chick embryo has been reported (Simard et al, 2006). Claudin-3 (xcla) has been reported to affect left-right patterning in the heart and gut in Xenopus (Brizuela et al., 2001), and similar results have been found for claudin-4 and -5 (unpublished data). However, little information exists on claudin proteins in the epithelium of developing chick intestine.

Rapid functional differentiation of the intestinal epithelium occurs between days 14 and 21 (the week before hatching), including formation of villi, microvilli and the terminal

web, changes in cell shape, and a dramatic increase in the density of goblet cells within the epithelium (Black, 1978; Black and Smith, 1989; Uni et al., 2003). Here the localization of claudin-3, -5, and -16 proteins were reported during the third week of embryonic development in the intestinal epithelium. Claudins-3 and -5 were chosen in order to compare the expression of these proteins in the chick intestine with that reported for mouse and rat intestine. Claudin-16 was of interest due to its presence in kidney epithelium where it is reported to function in paracellular transport of bivalent cations (Simon et al., 1999). In addition, the chick intestinal mRNA levels of claudin-1, -3, -5, -16, ZO-2, and TGFβ3 among three stages: 18 and 20 days of incubation and after 2 days of hatch were investigated. Developing chick intestine provides a dynamic model to investigate claudin localization at different embryonic ages, as well as in younger vs. older villi at the same age and along the crypt-to-villus tip axis of a single villus in hatched-chicks.

LITERATURE REVIEW

Embryonic development of the chick intestine:

Primitive gut tube formation is initiated by the invaginations of the ventral gut endoderm from the rostral (anterior) and caudal (posterior) ends of the embryo. These invaginations are called anterior and posterior intestinal portal, respectively. While these invaginations migrate toward each other, they form a simple tube behind them (Gruenwald, 1941). While the foregut and hindgut originate from the rostral and caudal invaginations, respectively, the midgut is derived from both. Foregut refers to the anterior part of the body from the mouth to the duodenum part of the intestine while midgut and hindgut covers the rest of the intestines in mature organisms (Carlson, 1999).

The luminal epithelial layer of the gut develops from the embryonic endoderm, and the other cell layers of the gut are derived from the splanchnic mesoderm layer that surrounds the endodermal epithelium. Specification of the gut involves the interaction between the endoderm-derived epithelium and the overlying mesoderm; and consequently, the luminal epithelium undergoes specification along the A-P axis to form esophagus, stomach, small and large intestines (Roberts, 1999). Meanwhile, the splanchnic mesoderm differentiates into smooth muscle layers in an organ specific manner. Several sequential signaling molecules have been identified in the crosstalk between the endoderm and mesoderm for the regionalization of the gut: firstly, Sonic hedgehog (SHH) is expressed by the endoderm and secreted in different concentrations and causes the differentiation of the adjacent mesoderm. Shh is believed to induce the activation of Hox genes (Hoxd, for example) in the mesoderm for its specification. Differentiated mesoderm then sends

paracrin type signals such as BMPs (BMP4, for example) and FGFs to endoderm for its specification. Further cross-talk between these two layers in a paracrin manner leads to differentiation of organs along AP axis of the gut. Liver, pancreas, and gallbladder are other gut organs, and they are also lined by the endoderm layer (Haffen et al., 1987; Kedinger, 1986, 1988).

Tight Junctions (TJs) and Claudin (cldn) Proteins:

Simple columnar epithelium of the intestine forms an active barrier between the lumen and the blood supply, and regulates absorption and secretion events of the intestine Therefore, the activity of intestinal epithelium is one of the key elements for intestinal homeostasis. Tight junctions are specialized, multi-component structures located at the most apical side of lateral cell membranes (Fig. 2.1) (Anderson and Van Itallie, 2008), and can be considered connections (kissing points) of adjacent cell membranes which are formed by the interactions of extracellular loops of opposing integral membrane proteins of two adjacent epithelial cells (Krause et al., 2008). Tight junctions are responsible for the sealing the spaces between the epithelial cells (the barrier function of tight junction); however, they are complex and dynamic structures, and play an important role in movement of molecules between the lumen and the capillary vessels by regulating the diffusion between the cells so called paracellular transport (Schneeberger and Lynch, 2004; Gonzales-Mariscal et al., 2003).

Cldn protein family is the main component of tight junction (TJ) strands and directly involved in the barrier function of TJs (Furuse et al., 1998; Furuse et al., 1996; Van Itallie and Anderson, 2006; Sonoda *et al*., 1999). In addition, the cldn protein family with

more than 20 members creates charge and size selective pores in the paracellular pathways and has a critical influence in the composition of the transported solutes in a tissue specific manner (Van Itallie and Anderson, 2006; Colegio et al., 2002; Rahner et al., 2001; Simon et al, 1999).

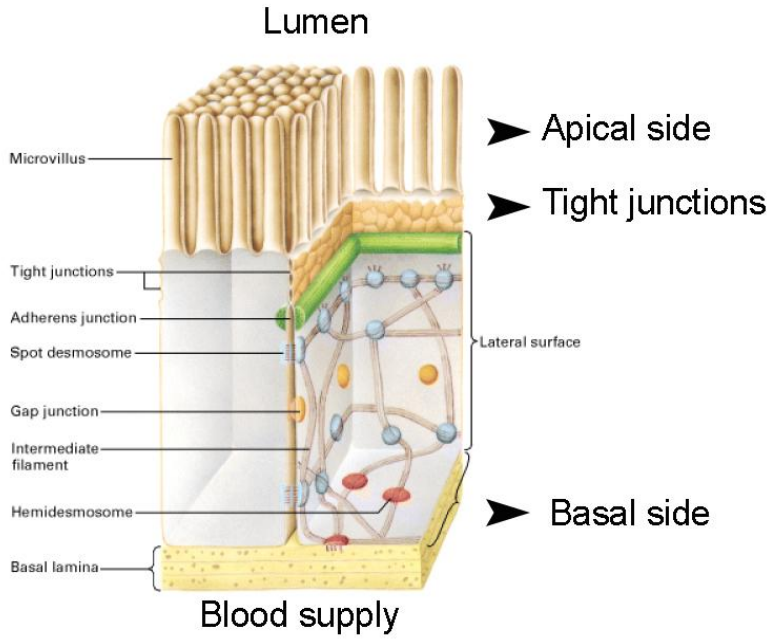


Figure 2.1: Localization of tight junctions in a typical epithelial cell. Tight junctions are located in most apical side of lateral cell membranes. Figure is based on: Lodish et al., 2000.

Claudins During Embryonic Development:

In addition to contributing to the barrier function of TJs in mature tissues, these junctions are also present and required for normal embryonic development of epithelial sheets (Simard et al, 2006; Turksen and Troy, 2001; Sonoda et al., 1999; Brizuela et al., 2001). Brizuela et al., (2001) reported the importance of the Xcla TJ protein in the regulation of cell-cell interactions during establishment of LR axis asymmetry of the heart,

gut and/or gall bladder during *Xenopus* development. In addition, it has been reported that Claudin-1 and -3 are expressed as early as Hamburger and Hamilton (HH) stage 4 and 6 chick embryos (Simard et al, 2006 and 2005; Haworth et al, 2005). Whole-mount in situ hybridization of cldn-1 mRNA in early embryos has revealed that cldn-1 was expressed throughout the ectoderm in stage 4-6 embryos (Simard et al, 2005). During these stages, cldn-1 expression was not detected in the notochord, developing heart, and the lateral plate mesoderm (Simard et al., 2006). Real-time PCR studies have revealed that cldn-1 mRNA was 1.4 fold higher on the left than the right sides of embryos at HH stage 4 (Simard et al., 2006). At later stages, cldn-1 was observed in the epithelia of nasal placode, pharyngeal arches, and the otic vesicle. At stage 16-18, cldn-1 expression was seen in primordial germ cells, developing lung epithelium, gut and mesonephros (Simard et al, 2005). At stage 20, cldn-3 expression was detected in the mesonephric system, liver, lung bud, and intestine at stage 20 chick embryos (Haworth et al, 2005).

Simard et al, 2006 reported the importance of the cldn-1 protein for the correct LR patterning of the heart looping in the chick embryo. Retroviral over-expression of cldn-1 caused an abnormal (leftward) heart looping without affecting the endogenous expression of main asymmetry related genes like Pitx2, Nodal, Shh, and Lefty-1 when these investigators injected retrovirus on the right side of the chick embryo at stage 4.

Claudin-6 has been reported to be present mainly in the embryonic tissues. It is expressed in mouse embryonic stem cells and embryonic kidney tubules and may have a role in the maturation of epidermis or paracellular permeability (Turksen and Troy 2001; Abuazza et al., 2006).

During embryogenesis cldns might provide a sealing role between the cells, regulate the passage of ions, and mediate signaling molecules and/or transcription factors. Secondly, these proteins might provide significant adhesions between the cells and might play a role in time and space specific cell migration or affect three dimensional configurations of the cells; and thirdly, expression of cldns might up- or down-regulate the expression of other genes related to development such as LR asymmetry genes (Levin, 2006; Simard et al, 2006; Brizuela *et al*., 2001; Tsukita and Furuse, 2000).

MATERIALS and METHODS

Animals:

Broiler-type 1-day old chicks, 6-week old chickens, and fertile eggs were obtained from the Poultry Farm of North Carolina State University. Eggs were incubated at 99.5° F in a forced-draft incubator for the indicated number of days. Adult mice were obtained from Biological Resources Facility of North Carolina State University.

Tissue Preparation for Staining:

Chicks and mice were sacrificed, and the small intestine was immediately removed. Tissue pieces approximately 0.5cm long were taken from the proximal end of the duodenal loop, from the midpoint of the jejunum, and from the midpoint of the ileum (Fig. 2.2). Pieces were rinsed with cold phosphate buffered saline (PBS), pH 7.4 to remove blood and debris, split open to expose the intestinal lumen, fixed in fresh, buffered 2% paraformaldehyde for two hours, and then frozen with liquid nitrogen. Cryosections of 6µm

were prepared on a cryostat vibratome (Bright OTF5000 – Bright Instrument Co Ltd.) at -16ºC.

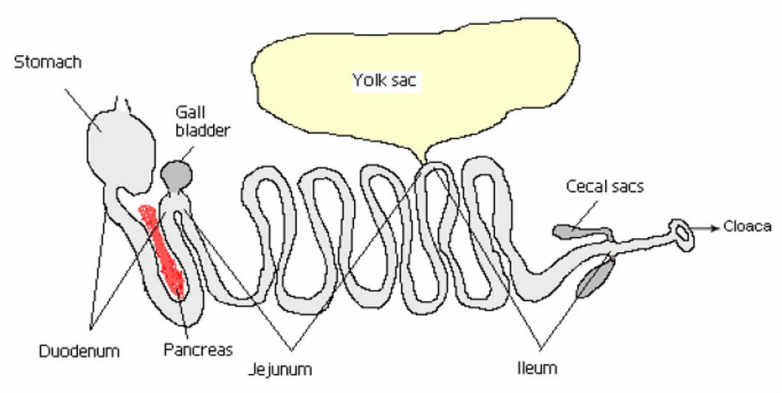


Figure 2.2: Schematic representation of duodenum, jejunum and ileum parts of the chicken intestine. For protein and gene expression analyses, duodenum part of the intestine was utilized.

Immunostaining:

Endogenous peroxidase activity was suppressed by treatment with 3% hydrogen peroxide for 30 min. Blocking solution was then applied for 1 h at room temperature (Vectastain kit, Vector labs, Burlingame, CA). Sections were next incubated with rabbit polyclonal claudin-3, claudin-5, or claudin-16 antibodies (Zymed, San Francisco, CA, USA) at 1:50 dilutions containing 0.3% TritonX-100 overnight at 4ºC in a closed and moistened chamber. For negative controls, duplicate sections were treated with normal serum for the same time and temperature. Diluted biotinylated universal IgG (Vectastain kit, Vector labs, Burlingame, CA) was applied and slides were incubated for 30 min at room temperature. Vectastain ABC reagent was then added for 30 min at room temperature. To visualize the claudin proteins, 3, 3'-diaminobenzidine Tetrahydrocloride (DAB, Sigma Labs, St. Louis, MO) was prepared according to the manufacturer's instructions and applied

to sections for 10 min. The DAB reaction was terminated by dipping the slides into water and sections were counterstained with hematoxylin. Stained sections were imaged by video microscopy using an Olympus BH2-RFCA microscope equipped with a Sony V500 digital camera.

Alcian Blue Staining:

Sections were stained in 1% alcian blue in 3% acetic acid (pH: 2.5) for 5 min and counterstained with eosin.

Western Blotting:

Tissue segments from each part of the chick intestine were homogenized in PBS. The homogenate was centrifuged at 1,000g for 20 min at 4ºC and re-suspended in PBS. Protein content of the homogenate described above were measured by a micro-assay based on the BCA protein assay (Pierce, Rockford, IL). Final concentrations of sample buffer and reducing agent (NuPAGE, Invitrogen, San Diego, CA) in the loaded samples were 141mM Tris base, 106mM Tris HCl, 73mM LDS, 0.5mM EDTA and, 50mM 1,4-dithiothretiol, Glycerol (8% v/v), serva blue G250 (0.019% w/v), and phenol red (0.006% w/v) were added prior to heating at 80ºC for 10 min. Proteins in 15 µg samples were separated by gel electrophoresis using 4–12% bis-tris gels (NuPAGE system) and MES/SDS-buffer (in mM: 50 2-(*N*-morpholino) ethanesulfonic acid, 50 Tris, 3.5 SDS, 1 Na2-EDTA; with addition of NuPAGE antioxidant) at 200 V (Xcell II SureLock; Invitrogen). Molecular size was estimated by including a prestained marker (Biorad, Hercules, CA). Following electrophoresis, the gel was blotted on to nitro-cellulose membranes (0.45 µm; Invitrogen) by submerged-blotting for 1 h at 30 V (XCell II; Invitrogen) in transfer buffer consisting of

25mM Tris, 192mM glycine, and 10% methanol. Membranes were blocked for 30 min in a 1:1 combination of TBS-T and LI-COR blocking buffer (Lincoln, NE) and washed in TBS-T. Membranes were incubated overnight at 4ºC with rabbit polyclonal claudin-3, claudin-5 (Zymed, San Francisco, CA, USA) and claudin-16 (Aviva System Biology, San Diego, USA) primary antibodies. Following washing, membranes were incubated 1 hour with goat anti-mouse and/or anti-rabbit secondary antibodies conjugated to Alexa IRDye 680 or IRDye 800CW (LI-COR). Blotted proteins were detected and their relative abundance quantified using the Odyssey infrared imaging system (LI-COR).

Isolation of Epithelial Cells from Intestinal Villi:

Isolation of epithelial cells from intestinal villi was performed as described by Black (1988). Briefly, tissue pieces removed from the small intestine were incubated in citrate buffer for 20 minutes at room temperature. Then, they were transferred to an EDTA-containing buffer and incubated at 37ºC with shaking for 30 minutes. The resulting cell suspension was filtered through silk screen to remove tissue debris and centrifuged for 5 min to obtain a pellet of epithelial cells. The cells were re-suspended in PBS and utilized for real-time PCR analyses.

Total RNA Extraction:

Organ cultured tissues were homogenized in Trizol reagent (Gibco, Grand Island, NY) using a bead beater. In order to separate nucleic acid from other macromolecules, chloroform was added into homogenate, vortexed, and then centrifuged; the upper aqueous phase contained total RNA. Total RNA was precipitated in 100% ethyl alcohol and hydrated with diethylpyrocarbonate treated (DEPC) water. Concentration and purity of the

total RNA samples were measured using a Nanodrop spectrophotometer (ND-1000). The quality of the total RNA was further analyzed by 1.5% agarose gel electrophoresis. Only total RNA with sufficient purity (A260/280 > 1.8) was used for further analyses. TURBO DNase (Ambion, Austin, TX) was used to remove genomic DNA contamination according to the manufacturer instructions.

Quantitative Real-Time PCR Analysis:

Reverse transcription and amplification were performed in a thermal cycler (Eppendorf) using a High Capacity cDNA reverse transcription Kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Primers were designed using Beacon Designer Software purchased from IDT (Iowa, USA). The list of the primers and the expected sizes of produced amplicons are shown in Table 2.1.

Table 2.1: List of the real-time PCR primer sets, their sequences, and expected amplicon sizes:

Genes and accession #	Primer sequence (5' to 3')		Annealing temp., ^{o}C	Amplicon size (bp)
1. Cldn1 (NM_001013611)	Sense	GTCTGGTTGGTGTGTTTG	54	196
	Antisense	TTAACGGGTGTGAAAGGG		
2. Cldn2 (XM_420271)	Sense	CTCAGCCCTCCATCAAAC	56	162
	Antisense	TGCGTCTTCTCCTCTTAC		
3. Cldn3 (NM_204202)	Sense	AGCCCTCCATCTCAGCAG	56	185
	Antisense	TTCTCCGCCAGACTCTCC		
4. Cldn5 (NM_204201)	Sense	GTCCCGCTCTGCTGGTTC	56	84
	Antisense	CCCTATCTCCCGCTTCTGG		
5. Cldn16 (XM_426702)	Sense	TACGCCATTGATGTCTACG	53	125
	Antisense	GATAAGAAGCAGCCCAGTG		
6. ZO-2 (NM_204918)	Sense	GAAGCAGAGGTCGTAGTAGG	55	140
	Antisense	CTGTCCATAGCCACCATCC		
7. TGFβ3 (NM_205454)	Sense	AAGGCATTGACAGTGAAG	55	173
	Antisense	GAAGCAGTAGTTGGTATCC		

To investigate the expression of claudins during development, SYBR green real-time PCR analysis was used. Reactions consisted of Power SYBR green PCR master mix (Applied Biosystems), forward and reverse primers at 10μm, and 2.5ng/ml cDNA sample. A real-time PCR protocol at 95ºC for 7 min followed by 50 cycles of 95ºC for 30 sec, 55ºC for 30 sec, and 72ºC for 30 sec was used (Fig. 2.3). Following amplification, a melting curve analysis was performed in 0.5ºC intervals. Cycle threshold (Ct) values were obtained using Icycler Software, duplicate values of each cDNA were averaged, and relative fold changes were determined using Pfaffl method which includes corrections for PCR efficiencies (Pfaffl, 2001). 18S rRNA was used as an internal control. Experimental errors were calculated by the standard error of the mean (SEM) of normalized Ct values from each development stage for individual genes, and were indicated as error bars in the graphs. Holm-Sidak test in conjunction with one way ANOVA statistical analysis was performed to determine the significance between treatment groups (Systat Software). P value < 0.05 was accepted as statistical significance.

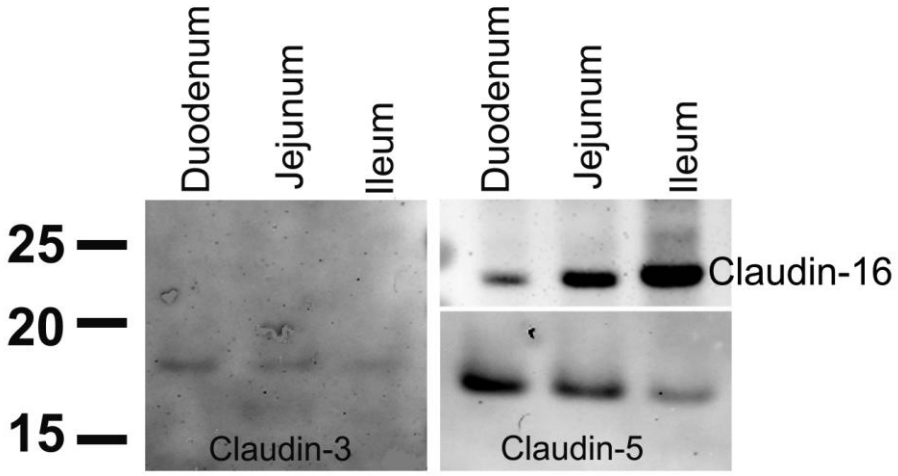
Cycle 1: (1X)
Step 1: 95.0ºC for 07:00
Cycle 2: (50X)
Step 1: 95.0ºC for 00:30
Step 2: 55.0ºC for 00:30
Step 3: 72.0ºC for 00:30
Cycle 3: (1X)
Step 1: 72.0ºC for 05:00
Cycle 4: (1X)
Step 1: 95.0ºC for 01:00
Cycle 5: (1X)
Step 1: 55.0ºC for 01:00
Cycle 6: (80X)
Step 1: 55.0ºC for 00:30
Increase setpoint temperature after cycle 2 by 0.5ºC

Figure 2.3: The real-time PCR protocol. Annealing temperature was adjusted according to each primer set.

RESULTS

Localization of claudin proteins in intestinal epithelium of post-hatch chicks:

Claudins-3, -5, and -16 were present in the intestine of 1-day old hatched chicks based on the results of western blotting (Fig. 2.4). While claudins -3 and -5 were present in higher amounts within duodenum as compared to the ileum, claudin-16 was higher in the ileum.



	claudin-3			claudin-5			claudin-16		
	D	J	I	D	J	I	D	J	I
Band intensities:	280	135	131	1914	1317	658	1148	2188	2667
Relative D in %:	100	48.2	46.8	100	68.8	34.4	100	190.6	232.3

Figure 2.4: Western blot analysis of claudin-3, 5, and 16 in duodenum, jejunum and ileum. Fifteen micrograms of protein was loaded in each lane. Relative band densities of cldn-3, 5, and 16 were indicated in duodenum, jejunum, and ileum.

Next, the distribution of these three claudin proteins within the epithelium was evaluated by an immunostaining technique (based on ETD by Ozden, 2004). As shown in

Fig. 2.5, these claudin proteins displayed different immunoreactivity patterns. Within the duodenum, claudin-3 staining was seen within both the apical and basal regions of the epithelial cells and occasionally between cells (Figs. 2.5B and B'), claudin-5 was located between epithelial cells, mainly in the tight junctional regions (Figs. 2.5C and C'), and claudin-16 was present in a subset of epithelial cells (Figs. 2.5D and D'). The shape of the stained regions and the distribution of the latter cells within the epithelium suggested that they were goblet cells. Alcian blue staining of goblet cells in sections of the same tissue provided confirming evidence that claudin-16 was within goblet cells of the epithelium (described below). Since the apparent presence of claudin-16 within intestinal goblet cells was unexpected, the immunostaining procedure was repeated on intestinal sections from older (6 week) chicks and from adult mice. In both cases, claudin-16 staining appeared to be present within goblet cells although staining was much lighter in mouse intestine (Figs. 2.8H and I).

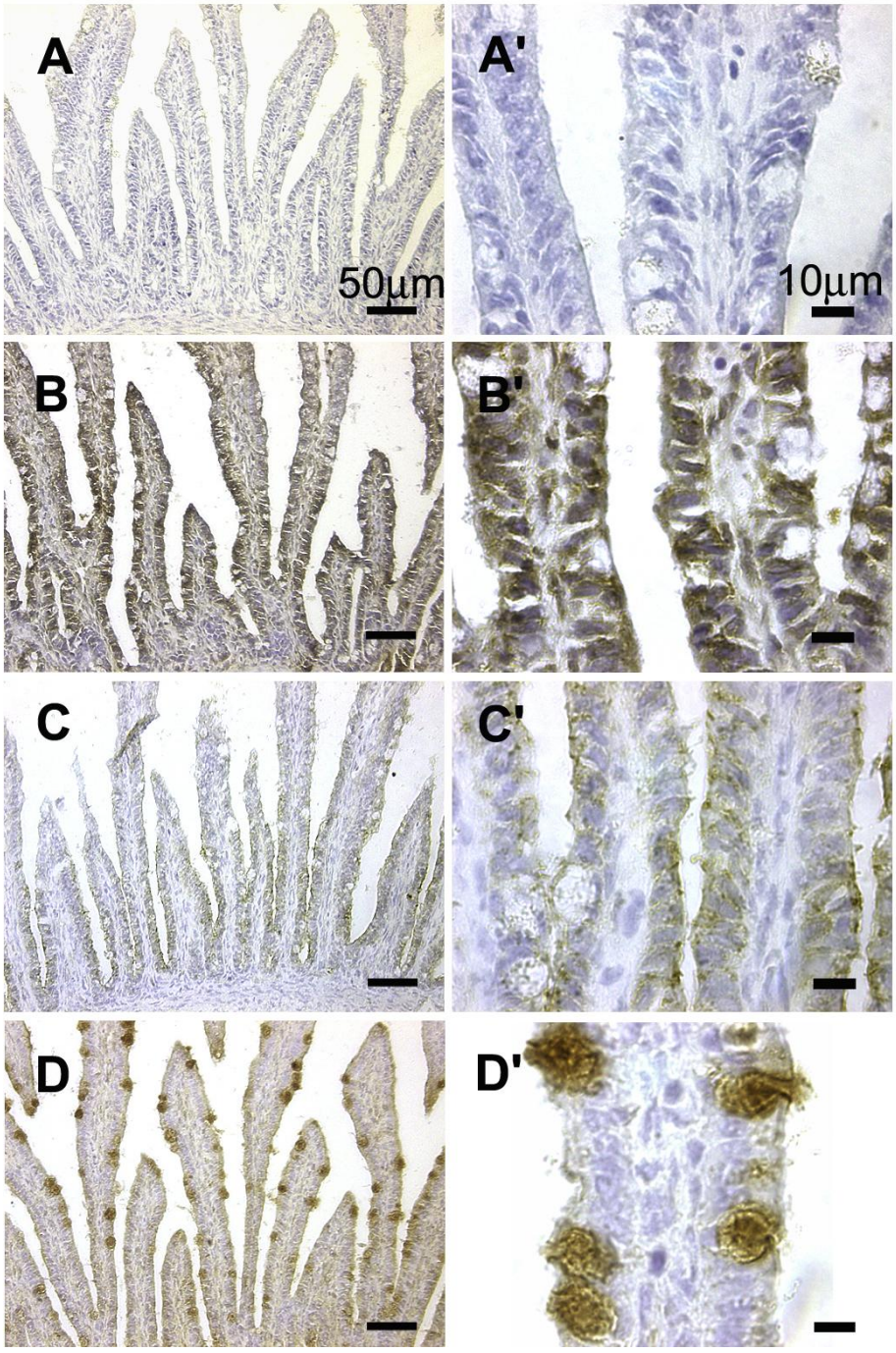


Figure 2.5: Localization of claudin- 3, 5, and 16 immunoreactivity in the duodenum of 1-day old hatched chicks. Left column (low power magnification), right column (high power magnification). (A, A'), there is no immunostaining in the negative controls. (B and B'), claudin-3 is located in basolateral membranes. (C and C') claudin-5 is in the epithelial junctional areas. (D and D') claudin-16 immunoreactivity in goblet cells. Based on Ozden, 2004.

Localization of claudin-3 proteins during embryonic development:

On day 12 of incubation, the duodenal epithelium stained with claudin-3 antibody (Fig. 2.6A). Throughout the duodenum, staining in epithelium of the upper villus region was stronger than that of villus base. On day 14, claudin-3 staining was similar, but more intense as compared to day 12; villus tips were again stained more strongly than the villus bases (Fig. 2.6B). On day 16, staining was similar to day 14, except the villus tip to base gradient was less apparent (Fig. 2.6C). On day 18, claudin-3 staining was strongest at the villus tips (Fig. 2.6D), but the day before hatching (day 20), staining was uniform throughout the epithelia layer from villus base to tip (Fig. 2.6E). During days 12-18 of development, the claudin-3 expression pattern within the jejunum and ileum was similar to that of duodenum, except that expression of claudin-3 protein within the ileum lagged two days behind that of the duodenum (data not shown). By 20 days, there was no obvious difference in claudin-3 staining in the three intestinal regions was found (data not shown). In 1-day old hatched chicks, claudin-3 staining was present along the entire length of most villi without showing an obvious gradient (Fig. 2.6G), but was weak within the crypt region (Fig. 2.6H).

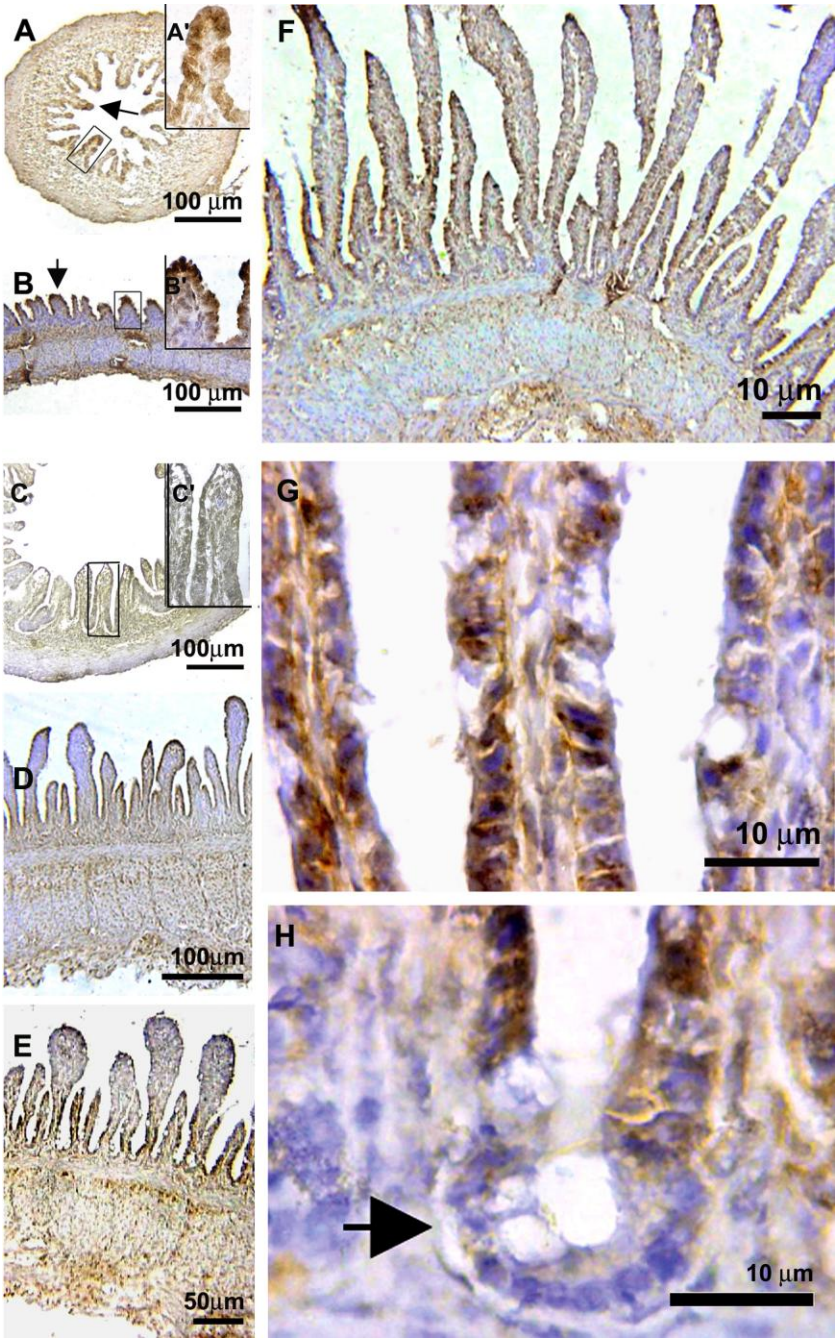


Figure 2.6: Claudin-3 staining in pre-hatch and post-hatch chick duodenum. A, 12- day embryo. A', high magnification of square in A. B, 14-day embryo. B', high magnification of square in B. C, 16-day embryo. C', high magnification of square in C. D, 18-day embryo. E, 20-day embryo. F, G, and H, 1-day old hatched chicks. G, basolateral staining is present in the lower half of a mature villus (arrow). No detectible claudin-3 was found in the crypt region of a villus (arrow) in H. Based on Ozden, 2004.

Localization of claudin-5 proteins during embryonic development:

At days 12 and 14 of incubation, claudin-5 staining of duodenum was detected within the cytoplasm of the epithelium, and tended to be more concentrated in the apical region of cells (Fig. 2.7A). No obvious gradient along the villus was found. On days 16, 18, and 20, claudin-5 staining remained within the apical regions of epithelium, but by day 20 could also be found in tight junctional regions (Figs. 2.7C, D, and E). Claudin-5 protein staining of the jejunum and ileum from 12-20 days was similar to that of the duodenum (data not shown). In 1-day old hatched chicks, claudin-5 staining was prominent in the tight junctional region of crypt and lower villus epithelium, but was less conspicuous in the upper regions of the longest (most mature) villi. In the shorter (less mature) villi, staining was uniform from villus base to tip (Fig. 2.7F and G). Staining was also seen within the endothelium of mucosal blood vessels (Fig. 2.7H).

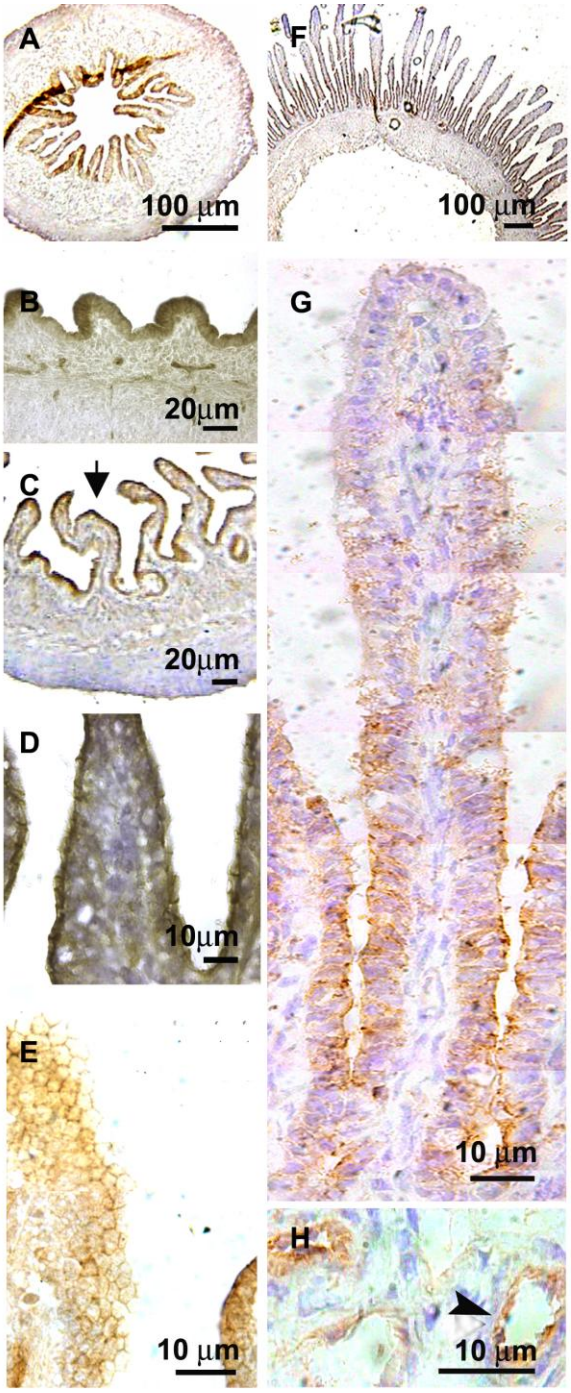


Figure 2.7: Claudin-5 staining in pre-hatch and post-hatch duodenum. A, 12-day embryo. B, 14-day embryo. C, 16-day embryo. D, 18-day embryo. E, 20-day embryo. F, G, and H are from 1-day old hatched chick duodenum. Arrowheads indicate endothelial staining in H. Based on Ozden, 2004.

Localization of claudin-16 proteins during embryonic development

Claudin-16 immunostaining of the goblet cells was first seen in the duodenum at 20 days of embryonic development; stained cells were located near villus tips (Fig. 2.8D). By 1-day post-hatch, claudin-16 positive goblet cells were present throughout most of the duodenal epithelium, but were absent from the villus base and crypt regions. This pattern was more pronounced in jejunum and ileum of 1-day old chicks; immunostained cells were most numerous near villus tips and rarely seen in the lower half of long villi (Figs. 2.8A, B, and C). Short villi of all three intestinal regions resembled the villi of 20-day embryos in that stained cells were few in number and located near villus tips.

The distribution of goblet cells within the intestinal epithelium was also visualized by staining with alcian blue. Comparison of sections stained with alcian blue vs. claudin-16 antibody revealed a similar distribution of stained cells in the upper villus from all three intestinal regions (Figs. 2.8A, B, and C). Although the presence of goblet cells within the crypts and lower villus region was demonstrated by alcian blue, these cells apparently did not contain claudin-16 protein.

In intestine from hatched chicks, mucus appeared to the emerging from some immunostained cells (Fig. 2.5D'), further confirming their identity as goblet cells. In some goblet cell images, claudin-16 protein staining appeared to be localized on the membranes of the mucin granules (Fig. 2.8E). Traces of immunostaining were also detected between epithelial cells, in the tight junctional region, and on the epithelial surface (Fig. 2.8G). The

latter staining can be attributed to secreted mucus that clings to the villus surface.

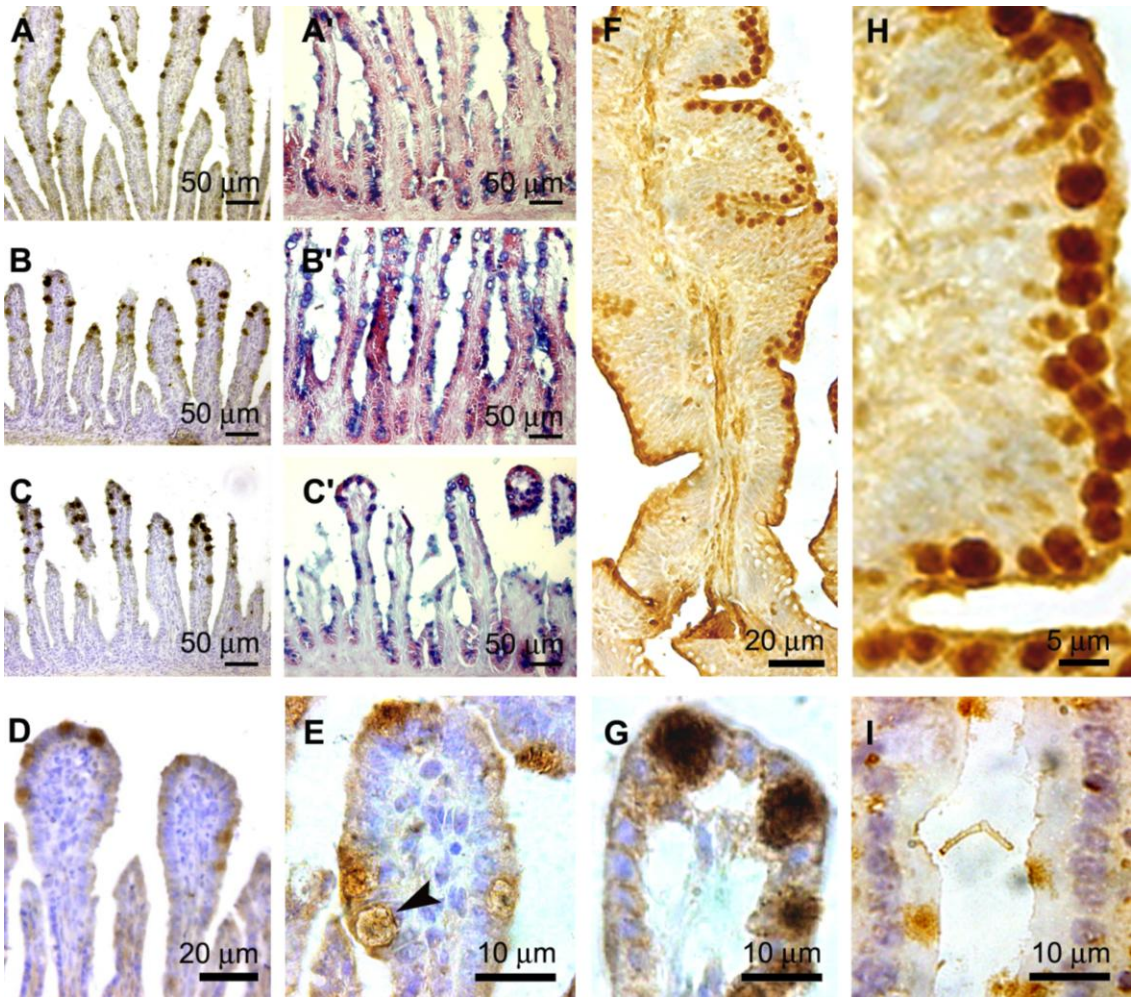


Figure 2.8: Claudin-16 staining in pre-hatch and post-hatch intestine. A, A'- C, C' are 1-day old chick intestine. A', Goblet cells were visualized with alcian blue in duodenum. (B') in jejunum, and C' in ileum. D, 20-day embryo duodenum. E, claudin-16 appeared to be localized on the mucin granule membranes. G, some claudin-16 staining was present between the epithelial cells in 1-day old hatched chick duodenum. Claudin-16 immunoreactivity is observed in goblet cells in 6-week adult chick intestine duodenum in F and H. I, claudin-16 immunoreactivity is seen in the goblet cells of adult mouse duodenum. Based on Ozden, 2004.

In summary, in both hatched and embryonic chick intestines, these three proteins localize to different areas of the intestinal epithelium. Claudin-3 was observed mainly in the apical and basal sides of the epithelial cells and in the cytoplasm. Claudin-5 was seen mainly in the epithelial tight junctional areas. Claudin-16 is present in the goblet cells on the more regions of villi (Fig. 2.9).

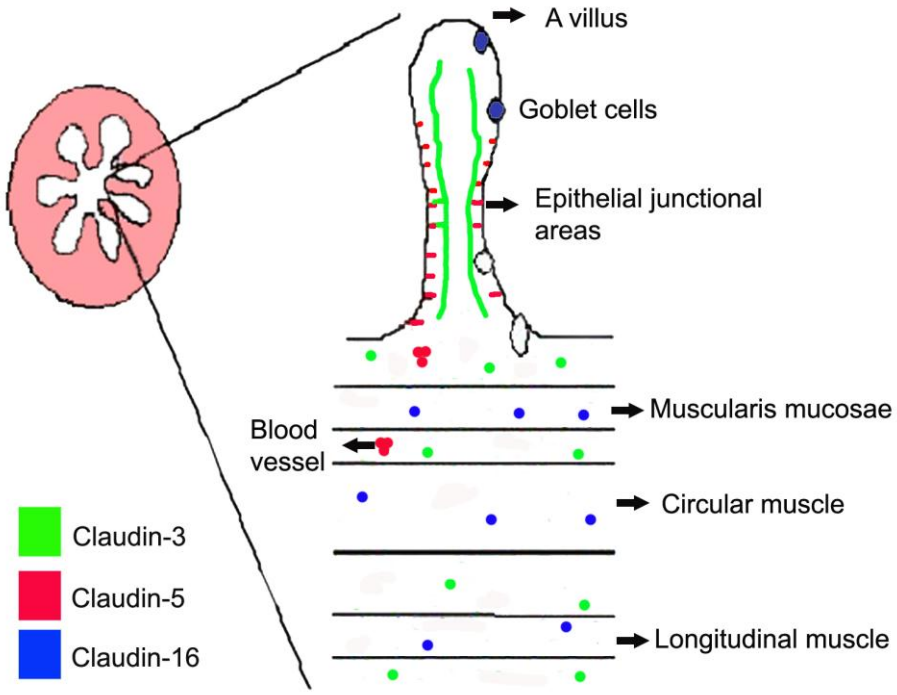


Figure 2.9: Summary for claudin-3 and -5 and -16 protein expressions in the intestine. Claudin-3, -5 and -16 proteins are present in both 1-day old hatched chick and embryonic chick intestines during one week before hatching. Based on Ozden, 2004.

Gene expression of claudins during embryonic development

To determine the expression profile of claudin mRNAs in the chick duodenal epithelium during embryonic development, SYBR-Green real time PCR analyses was used.

The expression levels of claudin-1, -2, -3, -5 and -16, TGFβ and ZO-2 in three developmental stages: 18 and 20 days of development, and 2 days post-hatch were compared. Relative gross expression patterns of the three claudin transcripts were similar at these developmental stages. With the exception of claudin-2, claudin expressions were lowest at 18 days of incubation and reached their highest levels on the day before hatching (20 days of embryonic development). Some decrease in the expression of these claudins at 2 days post-hatch was observed, but levels remained higher than in 18 day embryos (Figs. 2.10, 12, 13, 14)

Claudin-1 expression was the lowest among three stages examined. The day before hatching, its expression sharply increased 11.5 fold. At two days old hatched chick intestine, the mRNA levels were 3.4 folds higher than 18 days of embryonic intestine (Fig. 2.10).

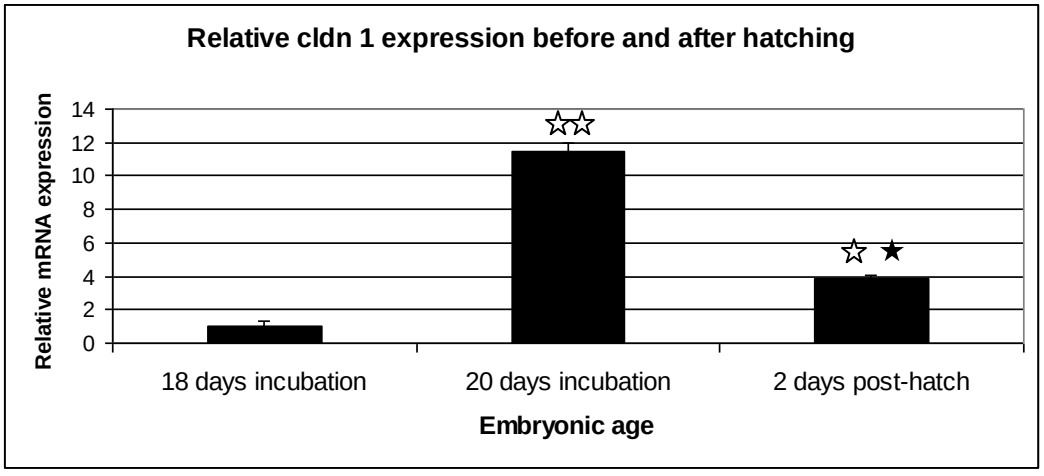


Figure 2.10: The expression levels of the claudin-1 mRNA of 20-day embryonic and hatched chick intestines relative to 18-day embryos. The data are reported as mean +/- SEM, n > or = 3, (☆☆- $p < 0.001$, ☆- $p < 0.05$ statistical significance compared to 18 days old embryos. "★" indicates statistical significance between 20-day embryo and 2-day hatched chick intestinal epithelia ($p < 0.05$).

The expression of **claudin-2** displayed a different pattern than other claudins that were examined. The expression levels of claudin-2 were similar between 18 and 20 days old embryonic intestines. The day after hatching, relative claudin-2 mRNA levels were two folds higher than during pre-hatch stages (Fig. 2.11).

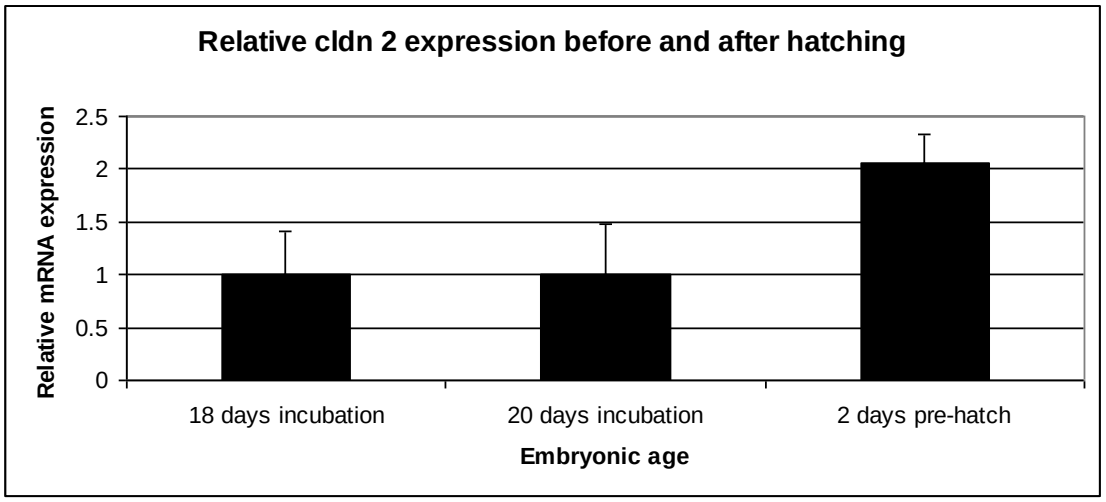


Figure 2.11: The expression levels of the claudin-2 mRNA of 20-day embryonic and hatched chick intestinal epithelia relative to 18-day embryos. The data are reported as mean +/- SEM, n = or > 3.

Claudin-3 expression at 18 days of incubation was relatively the lowest, and increased at 20-day pre-hatch and 2 days old post-hatch ages. Its expression reached its peak the day before hatching with an approximately 9.7-fold increase. After hatching, its expression lowered to approximately 3.5 folds of 18-day pre-hatch stage (Fig. 2.12).

Claudin-5 expression was relatively low at 18-day embryonic incubation. The day before and after hatching its expressions were approximately 3.8 and 2.8 folds of 18 days old embryo, respectively (Fig. 2.13).

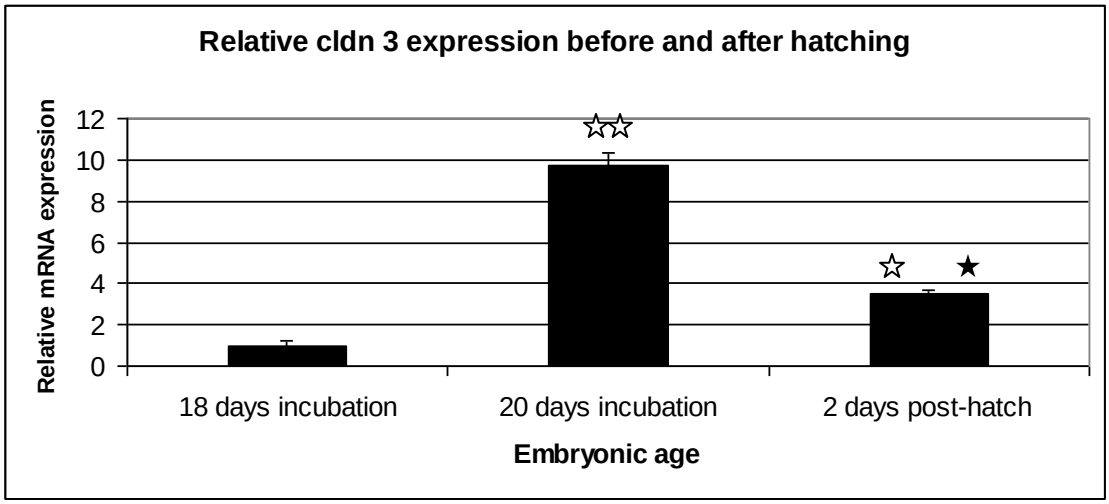


Figure 2.12: The expression levels of the claudin-3 mRNA of 20-day embryonic and hatched chick intestinal epithelia relative to 18-day embryos. The data are reported as mean +/- SEM, n = or > 3. (☆☆: $p < 0.001$, ☆: $p < 0.05$ statistical significance compared to 18 days old embryos. "★" indicates statistical significance between 20-day embryo and 2-day hatched chick intestinal epithelia ($p < 0.05$).

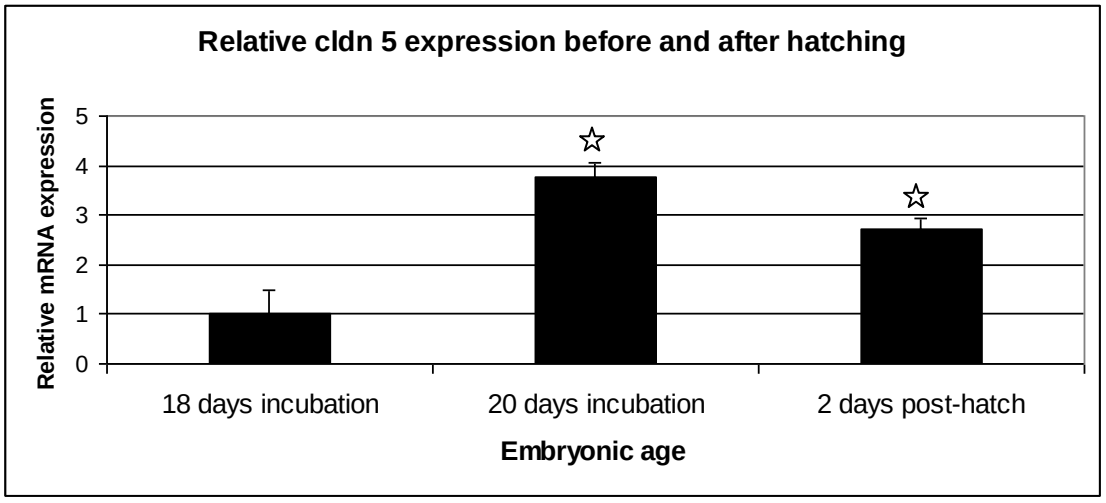


Figure 2.13: The expression levels of the claudin-5 mRNA of 20-day embryonic and hatched chick intestinal epithelia relative to 18-day embryos. The data are reported as mean +/- SEM, n = or > 3, ☆ : $p < 0.05$ compared to 18 days old embryos.

As for **claudin-16**, its expression reached its peak the day before hatching and was approximately 9.1 folds of 18 days old embryonic age. In two days post-hatching, there was some decrease relative to that of the day before hatching, approximately 3.7 fold more of claudin-16 mRNA expression was observed at that stage than that of the 18 day old (Fig. 2.14).

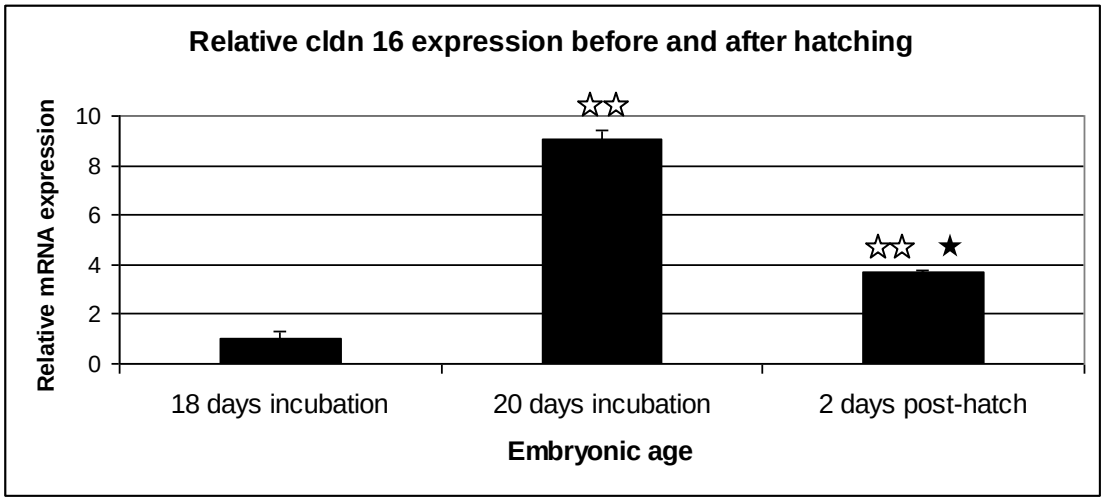


Figure 2.14: The expression levels of the claudin-16 mRNA of 20-day embryonic and hatched chicks relative to 18-day embryos. The data are reported as mean +/- SEM, n > or = 3, (☆☆- $p < 0.001$ compared to 18 days old embryos. ★ indicates statistical significance between 20-day embryo and 2-day hatched chick intestinal epithelia ($p < 0.05$).

The expression of another tight junction related protein was also examined and found to differ from that of the claudins. **ZO-2** expression was relatively the highest in 18-day embryos and declined at later developmental stages: the expression in 18-day embryos was 3.8 and 5.9 fold that of 20-day embryonic and 2-day hatched chick, respectively (Fig. 2.15).

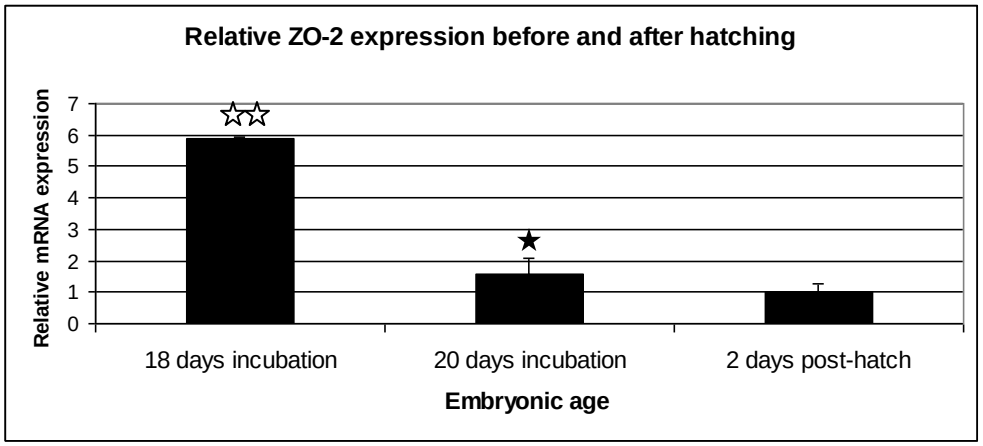


Figure 2.15: The expression levels of the ZO-2 mRNA of embryonic chicks relative to 2-day hatched chicks. The data are reported as mean +/- SEM, n > or = 3, (☆☆ - $p < 0.001$ compared to 2 days old chick. ★ - indicates statistical significance between 18 days old and 20 days old embryonic chick intestinal epithelia ($p < 0.05$).

The expression pattern of TGFβ3 was similar to that of ZO-2. Its expression was highest at 18 days old embryonic age and lowered smoothly as chicks develop. In 18 days old embryonic intestine, the expression levels of TGFβ3 were approximately 2.6 and 1.5 folds higher than 18 days old embryonic and 2 days old post-hatch stages (Fig. 2.16).

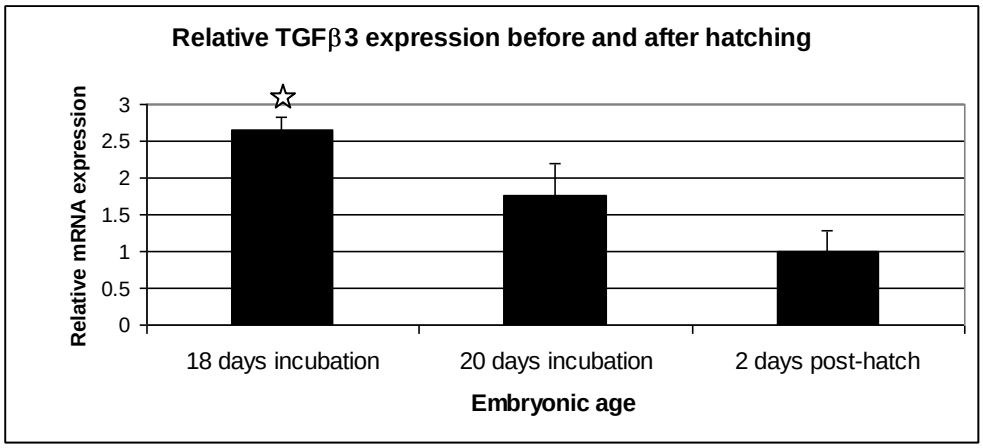


Figure 2.16: The expression levels of the TGFβ3 mRNA of embryonic chicks relative to 2-day hatched chicks. The data are reported as mean +/- SEM, n > or = 3; ☆ - $p < 0.05$ compared to 2 days old chick.

DISCUSSION

Protein and mRNA expression of three claudins were examined in the late pre-hatch and early post-hatch periods. Consistent with previous reports, some claudin tight junction proteins were detected in the chick intestine during embryonic development (Simard et al, 2006; Haworth et al., 2005).

Claudin-3 and -5 proteins were observed within the epithelial cytoplasm between 12 and 18 days of embryonic development. By 1-day post-hatch, these two proteins were concentrated in the epithelial basolateral membranes and apical junctions, respectively. Both claudin-3 and -5 protein expressions were high during pre-hatch and post-hatch periods. Their relative mRNA expressions increased between 18 and 20 days, then dropped. Relative transcript profiles of these two claudins were not inconsistent with protein profiles observed in immunostained sections from duodenum.

Claudin-3 is present along the gastrointestinal tract of mouse, rat, embryonic chick, and fish (Rahner et al., 2001; Holmes et al., 2006; Haworth et al., 2005; Bagherie-Lachidan et al, 2008). Claudin-3 overexpression has been reported to reduce paracellular permeability of human airway epithelia lines (Coyne et al., 2003). Conversely, reduction in claudin-3 expression caused increased paracellular permeability in Caco-2 intestinal cell line epithelia (McLaughlin et al., 2004). In the mouse kidney, claudin-3 was detected in the ascending loop of Henle, distally convoluted tubule, and collecting duct of nephrons where tight junctions are tighter, but its expression was absent in the proximal segment where tight junctions are leakier (Kiuchi-Saishin et al., 2002). Phosphorylation of cldn 3 with protein kinase A decreased TJ strength by decreasing TER and increasing TJ permeability in

ovarian cancer cell line OVCA 433 (D'Souza et. al., 2005). Additionally, a mutation in cldn3 mimicked the phoshorylation state of cldn 3 and caused an increase in paracellular flux and reduction in TER (D'Souza et. al., 2005). The majority of the reports suggest that cldn 3 has a tightening potential for TJ (Krause et. al., 2008).

In 1-day old chicks, claudin-3 protein expression was weak or absent in the crypt regions where intestinal stem cells are located and tight junctions are relatively leaky (Marcial et al., 1984). Claudin-3 staining was stronger in the villus epithelia where tight junctions are relatively tighter. Thus, these observations are consistent with most previous reports, and further supports the idea that claudin-3 "seals" the paracellular spaces in the intestine of young hatched chicks.

Claudin-5 has previously been reported in both junctional area and basolateral membranes of mouse intestine (Holmes at al., 2006), but is strictly localized to the junctional areas between epithelial cells in the rat intestine (Rahner et al., 2001). Our observations for this protein indicate that it is present mainly in tight junctional regions in 1-day old chick intestine. In contrast to claudin-3 protein expression, claudin-5 expression was strong in the crypt regions and lower parts of the villi in1-day old hatched chick. Intestinal crypt regions not only have leaky tight junctions, but also play a role in intestinal secretion (Marcial et al., 1984). Thus, this study suggests that claudin-5 in the crypt regions might function in regulating secretion though paracellular spaces. It has been reported that most proliferative cells in the chicken intestine are located in the crypt regions, and unlike the intestinal epithelial sheet of most mammals, some activity is also detected by proliferating cell nuclear antigen (PCNA) staining in the upper half of the villus from day

18 to day 36 post-hatch (Uni et al., 1998). In this study, strong expression of claudin-5 was seen in the lower parts of the villi, and it was very weak or absent in the upper half of the villi which seems similar to the proliferation pattern defined by Uni et al, 1998. Claudin-3 and -5 were proposed to affect the behavior and growth of various tumors. In gastric and esophageal tumors having high levels of apoptosis, proliferation indexes expressed significantly stronger levels of claudin-5 (Takala et al, 2007; Soini et al, 2006), support the idea that claudin-5 may be associated with proliferation in the chick intestine. Like claudin-5, claudin-3 was associated with high apoptosis index in the esophageal cancer (Takala et al., 2007). The association of these proteins with apoptosis and proliferation in the chick intestine is under investigation in our lab.

Claudin-16 has been reported to be function in magnesium and calcium transport through paracellular pathways in the thick portion of the loop of Henle (Simon et al., 1999). In addition, expression of claudin-16 has been reported in human salivary excretory ducts (Kriegs et al., 2007). Interestingly, claudin-16-like protein expression was detected mainly in the goblet cells beginning at 20 days of embryonic development. Immunostaining was most intense in the most mature goblet cells which are located at the tip of the villi in 1-day old and adult chick intestine. Claudin-16 mRNA expression showed a sharp peak at 20 days of embryonic age, the stage at which it was first detected claudin-16-like protein expression. In 2-day old post-hatch chicks, mRNA expression dropped more than 2 folds relative to the 20 day value, whereas protein expression greatly increased during this 3-day period. Increased translation rate of claudin-16 transcripts may be one of the explanations for increased protein but decreased mRNA levels at 2 days post-hatch. The increase in a

claudin-16-like protein within the goblet cells just after hatching may reflect the onset of mucus secretion as intestinal function begins. Two kinds of goblet cell secretions have been identified: baseline secretion and accelerated secretion. Baseline secretion is a slow and continuous exocytosis of individual mucin granules that is required for maintenance of the mucus layer. In accelerated secretion, mucin granules fuse to each other and to the plasma membrane, and all granules discharge mucin content through a pore in the plasma membrane in a very short time. It has been reported that Ca^{++} plays a crucial role in packaging of mucin molecules in the mucin granules of goblet cells, and a high amount of Ca^{++} is present in mature goblet cells (Takano and Akai, 1988; Warner and Coleman, 1975; Verdugo, 1990). Ca^{++} neutralizes the polyanionic charges of mucin molecules, and keeps them in compacted form. When this Ca^{++} shielding is lost, mutual repulsion of polyanionic charges of mucin molecules provides the driving force for mucin polymerization, and accordingly, a large volume of mucus is secreted in a very short time period in stimulated mucus secretion (Verdugo, 1990). In addition to its mucin secretion role, Ca^{++} plays an important role in the differentiation of goblet cells in the embryonic chick duodenum (Black and Smith, 1989). It is likely that Claudin-16 might play a role in "Ca^{++} related" mucus secretion of goblet cells just as previously proposed for cldn16 in stimulated salivary gland secretion by Kriegs et al., 2007. Further studies are needed to investigate the exact subcellular localization of claudin-16 and to determine its potential role in goblet cell function.

The transepithelial resistance (TER) of TJs can be increased or decreased by changing the expression profiles of claudins which change the ionic charge selectivity in

cultured epithelial monolayers. This suggests that claudins are bordering charge specific paracellular pores (Colegio et. al., 2002; Hou et al., 2005; Alexandre et al., 2007).

In general, claudins can be classified into two groups with respect to their cation permeability: claudins having tightening potential (claudin 1, 3, 4, 5, 11, and 14, for example), and claudins having pore forming claudins (claudin 2, 7, and 16, for example) in MDCK and LLC-PK1 cell lines (Amasheh et al., 2002; Wen et al., 2004; Alexandre et al., 2005; Van Itallie et al., 2001; Van Itallie et al. 2003). However, the tightening ability of individual claudin depends on the composition and abundance of other claudins that lines the paracellular pores in different tissues (Krause et. al., 2008; Yu, 2003).

Claudin-1 is a common tight junctional protein and expressed in numerous tissues (Van Itallie and Anderson, 2006). It has a crucial barrier function in mammalian epidermis. Claudin-1 knockout mice died due to severe dehydration soon after the birth (Furuse et al., 2002). Overexpression of cldn 1 enhances barrier properties of TJs in airway and kidney MDCK cell line (Coyne et al., 2003; Inai et al., 1999). In Caco2 small intestine epithelial cell line, rotavirus infection induced high paracellular permeability and decreased TER as a consequence of significant redistribution of claudin-1 (Dickman et. al., 2000). High levels of claudin-1 mRNA expression were detected especially the day before hatching. As proposed in various tissues, claudin-1 might contribute to barrier function of intestinal epithelia by tightening TJs.

On the contrary to claudin 1, 3, 4 and 5, **claudin 2** is believed to form leaky pores in TJs (Furuse et al., 2001; Amasheh et al., 2006). Introduction of claudin-2 expression into high-resistance MDCK cell line caused a decrease in TER (Furuse et al., 2001). This result

suggests that claudin-2 forms cation-selective pores in the MDCK cells (Amasheh et al., 2002; Furuse et al., 2001). Claudin 2 is expressed mainly in mouse intestinal crypts (Holmes et al., 2006), and claudin 2 expression displays a declining gradient from crypt to villus tip (Mariadason et al., 2005). In the crypt regions, the size of the pores in the paracellular spaces is around 50-60 A^0 in radii while in the villi, it is less than 6 A^0 (Fihn et al., 2000). Claudin 2 but not some other claudin expressions, such as claudin 4, 14, and 18, is suggested to increase the number of small pores with 4 A^o radii in the paracellular spaces in MDCK cell line without affecting the pore size (Van Itallie et al., 2008). The expression pattern of claudin-2 was different than other claudins examined. Since the proposed roles of claudin-2 are generally different from especially claudin-1, 3 and 5, the expression of claudin-2 is not surprising. In the hatched chick intestine, claudin-2 might have a role in increasing the number of small pores as previously proposed in MDCK cells (Van Itallie et al. 2008).

Zona occludens-2 (ZO-2) is one of the adapter proteins described in connecting tight junctions to the cytoskeleton (Itoh et al., 1999; Gonzalez-Mariscal et al., 2003). It is evolutionary conserved, and its protein binding domains show 92-100% similarity between chick and mammalian aminoacid sequences (Collins and Rizzolo, 1998). ZO-1 and ZO-2 are thought to initiate the correct localization of claudins and formation of tight junction structure (Umeda et al., 2006). Studies in ZO-2 knockout mice revealed that this protein is crucial for mouse embryonic development (Xu et. al., 2007). In this study, ZO-2 mRNA expression was detected in embryonic chick intestinal epithelium. Unlike claudin-3, -5 and -16, which showed a peak in 20-day embryos; expression of ZO-2 preceded the expression

of claudins and displayed a peak in 18 day old embryos. Its expression smoothly lowered as chicks develop. Further studies needed to elucidate the roles of ZO-2 on the correct orientation of claudins during development.

Transforming growth factor-beta (TGFβ) is a multifunctional cytokine that can be produced by the intestinal epithelia (Howe et al., 2005). Three major forms of TGFβ have been identified in both adult and embryonic intestine (TGFβ1, 2, and 3) (Thompson et al., 1989; Massague, 1990). Aminoacid sequences of TGFβ isoforms are more than 97% identical between some mammalian and avian species (Podolski, 1994). In avian, two additional forms were defined (TGFβ4 and TGFβ5). In general, the activity profiles of TGFβ1 and TGFβ3 are parallel to each other (Cheifetz et al., 1990). In the small intestinal epithelium, TGFβs play important roles in proliferation, differentiation, embryogenesis, and development. In addition, they play regulatory roles for epithelial-mesenchymal transition and TGFβ down-regulates claudin 1 expression in mature hepatocytes (Kojima et al., 2007). TGFβ protects epithelial barrier function in the intestine (Howe et al., 2005). It caused a significant increase in TER and decrease in paracellular mannitol flux by inducing claudin1 protein expression in T84 colonic epithelial cell line (Howe et al., 2005). In addition, it prevents the loss of claudin-2, occludin, and ZO-1 during *Escherichia coli* O157:H7 infection. In sertoli cells, TGFβ3 regulates TJ permeability by negatively regulating the expressions of occluding, cldn 11 and ZO-1 (Loi et al., 2001). So, the effects of various TGFβ isoforms on the expression of various claudins are different in different conditions and tissues. In this study, TGFβ3 mRNA levels decreased smoothly as the embryo develops

from 18 days pre-hatch to 2 days of post-hatch periods. Conversely, claudin 1, 3, 5, 16 mRNA levels were low in 18 days of pre-hatch period and peaked before hatching. If TGFβ3 is involved in the regulation of certain claudin expression in the chick intestine, it would be a negative regulator as reported in sertoli cells. Further studies needed to determine the effects of TGFβ3 on the regulation of tight junctions in the young chick intestine.

CONCLUSIONS

Expression of various claudins was detected in the intestinal epithelium during the last week of embryonic development. In addition to the known barrier and fence functions of claudins within the tight junctions of mature tissues, claudins might have additional roles in differentiation and maturation of the chick intestinal epithelia during late embryonic stages.

REFERENCES

Abuazza G, Becker A, Williams SS, Chakravarty S, Truong HT, Lin F, Baum M. 2006. Claudins 6, 9, and 13 are developmentally expressed renal tight junction proteins. Am J Physiol Renal Physiol. 291(6): F1132-1141.

Alexandre MD, Lu Q, Chen YH. 2005. Overexpression of claudin-7 decreases the paracellular Cl− conductance and increases the paracellular Na+ conductance in LLC-PK1 cells. J. Cell Sci. 118:2683–93

Amasheh S, Meiri N, Gitter AH, Schoneberg T, Mankertz J, Schulzke JD, Fromm M. 2002. Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells. J Cell Sci. 115:4969–4976.

Bagherie-Lachidan M, Wright SI, Kelly SP. 2008. Claudin-3 tight junction proteins in Tetraodon nigroviridis: cloning, tissue-specific expression, and a role in hydromineral balance. Am J Physiol Regul Integr Comp Physiol. 294(5):R1638-1647.

Black BL, Smith JE. (1989). Regulation of goblet cell differentiation by calcium in embryonic chick intestine. FASEB J.,3(14):2653-2659.

Black BL. 1978. Morphological development of the epithelium of the embryonic chick intestine in culture: Influence of thyroxine and hydrocortisone. Am. J. Anat. 153: 573-599.

Black BL. 1988. Development of glucose active transport in embryonic chick intestine. Influence of thyroxine and hydrocortisone. Comp Biochem Physiol A. 90(3):379-86.

Black, BL and Smith, JE. 1989. Regulation of goblet cell differentiation by calcium in embryonic chick intestine. FASEB J. 3: 2653-2659.

Brizuela BJ, Wessely O, DeRobertis EM. 2001. Overexpression of the Xenopus tight-junction protein claudin causes randomization of the left-right body axis. Dev Biol., 230(2):217-229.

Carlson B. 1999. Human Embryology and Developmental Biology. Mosby Yearbook, St. Louis, MO.

Cheifetz S, Hernandez H, Laiho M, ten Dijke P, Iwata KK, Massagué J. 1990. Distinct transforming growth factor-beta (TGF-beta) receptor subsets as determinants of cellular responsiveness to three TGF-beta isoforms. J Biol Chem. 265(33):20533-20538.

Collins JR, Rizzolo LJ. 1998. Protein-binding domains of the tight junction protein, ZO-2, are highly conserved between avian and mammalian species. Biochem Biophys Res Commun. 252(3):617-622.

Coyne CB, Gambling TM, Boucher RC, Carson JL, Johnson LG. 2003. Role of claudin interactions in airway tight junctional permeability. Am J Physiol Lung Cell Mol Physiol. 285(5):L1166-1178.

Dickman KG, Hempson SJ, Anderson J, Lippe S, Zhao L, Burakoff R, Shaw RD. 2000. Rotavirus alters paracellular permeability and energy metabolism in Caco-2 cells. Am J Physiol. 279:G757–G766.

Fujita H, Chiba H, Yokozaki H, Sakai N, Sugimoto K, Wada T, Kojima T, Yamashita T, Sawada N. 2006. Differential expression and subcellular localization of claudin-7, -8, -12, -13, and -15 along the mouse intestine. J Histochem Cytochem., 54(8):933-944.

Furuse M, Hata M, Furuse K, Yoshida Y, Haratake A, Sugitani Y, Noda T, Kubo A, Tsukita S. 2002. Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. J Cell Biol. 156:1099–1111

Furuse M, Sasaki H, Tsukita S. 1999. Manner of interaction of heterogeneous claudin species within and between tight junction strands. J Cell Biol., 147(4):891-903.

Furuse M, Tsukita S. 2006. Claudins in occluding junctions of humans and flies. Trends Cell Biol. 16(4):181-188.

González-Mariscal L, Betanzos A, Nava P, Jaramillo BE. 2003. Tight junction proteins. Prog Biophys Mol Biol. 81(1):1-44.

Gruenwald P. 1941. Normal and abnormal detachment of body and gut from the blastoderm in the chick embryo, with remarks on the early development of the allantois. J. Morphol. 69, 83–125.

Haffen K, Kedinger M, Simonassmann P. 1987. Mesenchyme-dependent differentiation of epithelial progenitor cells in the gut. Journal of Pediatric Gastroenterology and Nutrition 6:14-23.

Haworth KE, El-Hanfy A, Prayag S, Healy C, Dietrich S, Sharpe P. 2005. Expression of Claudin-3 during chick development. Gene Expr Patterns. 6(1):40-44.

Holmes JL, Van Itallie CM, Rasmussen JE, Anderson JM. 2006. Claudin profiling in the mouse during postnatal intestinal development and along the gastrointestinal tract reveals complex expression patterns. Gene Expr Patterns, 6(6):581-588.

Hou J, Shan Q, Wang T, Gomes AS, Yan Q, Paul DL, Bleich M, Goodenough DA. 2007. Transgenic RNAi depletion of claudin-16 and the renal handling of magnesium. J Biol Chem. 282(23):17114-17122.

Howe KL, Reardon C, Wang A, Nazli A, McKay DM. 2005. Transforming growth factor-beta regulation of epithelial tight junction proteins enhances barrier function and blocks enterohemorrhagic Escherichia coli O157:H7-induced increased permeability. Am J Pathol. 167(6):1587-1597.

Inai T, Sengoku A, Guan X, Hirose E, Iida H, Shibata Y. 2005. Heterogeneity in expression and subcellular localization of tight junction proteins, claudin-10 and -15, examined by RT-PCR and immunofluorescence microscopy. Arch. Histol. Cytol. 68(5):349-360.

Itoh M, Morita K, Tsukita S. 1999. Characterization of ZO-2 as a MAGUK family member associated with tight and adherens junctions with a binding affinity to occludin and a catenin. J. Biol. Chem. 274:5981–5986.

Kedinger M, Simonassmann P, Bouziges F, Haffen K. 1988. Epithelial mesenchymal interactions in intestinal epithelial differentiation. Scandinavian Journal of Gastroenterology 23:62-69.

Kedinger M, Simonassmann PM, Lacroix B, Marxer A, Hauri HP, Haffen K. 1986. Fetal gut mesenchyme induces differentiation of cultured intestinal endodermal and crypt cells. Developmental Biology 113:474-483.

Kiuchi-Saishin Y, Gotoh S, Furuse M, Takasuga A, Tano Y, Tsukita S. 2002. Differential expression patterns of claudins, tight junction membrane proteins, in mouse nephron segments. J Am Soc Nephrol. 13(4):875-886.

Kojima T, Takano K, Yamamoto T, Murata M, Son S, Imamura M, Yamaguchi H, Osanai M, Chiba H, Himi T, Sawada N. 2008. Transforming growth factor-beta induces epithelial to mesenchymal transition by down-regulation of claudin-1 expression and the fence function in adult rat hepatocytes. Liver Int. 28(4):534-45.

Krause G, Winkler L, Mueller SL, Haseloff RF, Piontek J, Blasig IE. 2008. Structure and function of claudins. Biochim Biophys Acta. 1778(3):631-645.

Kriegs JO, Homann V, Kinne-Saffran E, Kinne RK. 2007. Identification and subcellular localization of paracellin-1 (claudin-16) in human salivary glands. Histochem Cell Biol., 128(1):45-53.

Levin M. 2006. Is the early left-right axis like a plant, a kidney, or a neuron? The integration of physiological signals in embryonic asymmetry. Birth Defects Res C Embryo Today. 78(3):191-223.

Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, Darnell JE. 2000. Molecular Cell Biology. 4th ed. New York: W. H. Freeman & Co.

Lui WY, Lee WM, Cheng CY. 2001. Transforming growth factor-b3 (TGFb3) perturbs the inter-Sertoli tight junction permeability barrier in vitro possibly mediated its effects on occludin, zonula occludens-1, and claudin-11. Endocrinology. 142:1865–1877.

Marcial MA, Carlson SL, Madara JL. 1984. Partitioning of paracellular conductance along the ileal crypt-villus axis: a hypothesis based on structural analysis with detailed consideration of tight junction structure-function relationships. J Membr Biol. 80(1):59-70.

Mariadason JM, Nicholas C, L'Italien KE, Zhuang M, Smartt HJ, Heerdt BG, Yang W, Corner GA, Wilson AJ, Klampfer L, Arango D, Augenlicht LH. 2005. Gene expression profiling of intestinal epithelial cell maturation along the crypt-villus axis. Gastroenterology. 128(4):1081-1088.

Massagué J. 1990. The transforming growth factor-beta family. Annu Rev Cell Biol. 6:597-641.

McLaughlin J, Padfield PJ, Burt JP, O'Neill CA. 2004. Ochratoxin A increases permeability through tight junctions by removal of specific claudin isoforms. Am J Physiol Cell Physiol. 287(5):C1412-1417.

Morita K, Sasaki H, Furuse M, Tsukita S. 1999. Endothelial claudin: claudin-5/TMVCF constitutes tight junction strands in endothelial cells. J Cell Biol., 147(1):185-194.

Ozden O. 2004. Developmental profile of claudin-3, claudin-5, and claudin-16 tight junctions proteins in the chick intestine. Master thesis, ETD. North Carolina State University.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29(9):e45.

Podolsky DK. 1994. Peptide growth factors in the gastrointestinal tract. In: Johnson LR, editor. Physiology of the gastrointestinal tract. New York: Raven Press. p. 129-167.

Powell DW. 1981. Barrier function of epithelia. Am J Physiol., 241(4):G275-G288.

Rahner C, Mitic LL, Anderson JM. 2001. Heterogeneity in expression and subcellular localization of claudins 2, 3, 4, and 5 in the rat liver, pancreas, and gut. Gastroenterology, 120(2):411-422.

Roberts DJ. 2000. Molecular mechanisms of development of the gastrointestinal tract. Developmental Dynamics 219:109-120.

Schneeberger EE, Lynch RD. 1992. Structure, function, and regulation of cellular tight junctions. Am J Physiol., 262(6 Pt 1):L647-L661.

Simard A, Di Pietro E, Ryan AK. 2005. Gene expression pattern of Claudin-1 during chick embryogenesis. Gene Expr Patterns, 5(4):553-560.

Simard A, Di Pietro E, Young CR, Plaza S, Ryan AK. 2006. Alterations in heart looping induced by overexpression of the tight junction protein Claudin-1 are dependent on its C-terminal cytoplasmic tail. Mech Dev., 123(3):210-227.

Simon DB, Lu Y, Choate KA, Velazquez H, Al-Sabban E, Praga M, Casari G, Bettinelli A, Colussi G, Rodriguez-Soriano J, McCredie D, Milford D, Sanjad S, Lifton RP. 1999. Paracellin-1, a renal tight junction protein required for paracellular Mg2+ resorption. Science, 285(5424):103-106.

Soini Y, Tommola S, Helin H, Martikainen P. 2006. Claudins 1, 3, 4 and 5 in gastric carcinoma, loss of claudin expression associates with the diffuse subtype. Virchows Arch. 448(1):52-58.

Sonoda N, Furuse M, Sasaki H, Yonemura S, Katahira J, Horiguchi Y, Tsukita S. 1999. Clostridium perfringens enterotoxin fragment removes specific claudins from tight junction strands: Evidence for direct involvement of claudins in tight junction barrier. J Cell Biol., 147(1):195-204.

Specian RD, Neutra MR. 1980. Mechanism of rapid mucus secretion in goblet cells stimulated by acetylcholine. J Cell Biol., 85(3):626-640.

Takala H, Saarnio J, Wiik H, Soini Y. 2007. Claudins 1, 3, 4, 5 and 7 in esophageal cancer: loss of claudin 3 and 4 expression is associated with metastatic behavior. APMIS. 115(7):838-847.

Takano Y, Akai M. 1988. Histochemical, ultrastructural and X-ray microprobe analytical studies of localization of calcium in the mucous lining of the rat duodenum. Histochemistry,89(5):429-36.

Tipsmark CK, Madsen SS, Seidelin M, Christensen AS, Cutler CP, Cramb G. 2002. Dynamics of Na(+),K(+),2Cl(-) cotransporter and Na(+),K(+)-ATPase expression in the branchial epithelium of brown trout (Salmo trutta) and Atlantic salmon (Salmo salar). J Exp Zool., 293(2):106-118.

Tsukita S, Furuse M. 2000. The structure and function of claudins, cell adhesion molecules at tight junctions. Ann N Y Acad Sci. 915:129-135.

Turksen K, Troy TC. 2001. Claudin-6: a novel tight junction molecule is developmentally regulated in mouse embryonic epithelium. Dev Dyn. 222(2):292-300.

Umeda K, Ikenouchi J, Katahira-Tayama S, Furuse K, Sasaki H, Nakayama M, Matsui T, Tsukita S, Furuse M, Tsukita S. 2006. ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation. Cell. 126(4):741-754.

Uni Z, Platin R, Sklan D. 1988. Cell proliferation in chicken intestinal epithelium occurs both in the crypt and along the villus. J Comp Physiol [B]. 168(4):241-247.

Uni Z, Tako E, Gal-Garber O, Sklan D. 2003. Morphological, molecular, and functional changes in the chicken small intestine of the late-term embryo. Poult Sci. 82(11):1747-1754.

Van Itallie CM, Anderson JM. 2004. The molecular physiology of tight junction pores. Physiology (Bethesda). 19:331-338.

Van Itallie CM, Anderson JM. 2006. Claudins and epithelial paracellular transport. Annu Rev Physiol. 68:403-429.

Van Itallie CM, Holmes J, Bridges A, Gookin JL, Coccaro MR, Proctor W, Colegio OR, Anderson JM. 2008. The density of small tight junction pores varies among cell types and is increased by expression of claudin-2. J Cell Sci. 121(Pt 3):298-305.

Verdugo P. 1990. Goblet cells secretion and mucogenesis. Annu Rev Physiol., 52:157-176.

Warner RR, Coleman JR. 1975. Electron probe analysis of calcium transport by small intestine. J Cell Biol., 64(1):54-74.

Xu J, Kausalya PJ, Phua DC, Ali SM, Hossain Z, Hunziker W. 2008. Early embryonic lethality of mice lacking ZO-2, but Not ZO-3, reveals critical and nonredundant roles for individual zonula occludens proteins in mammalian development. Mol Cell Biol. 28(5):1669-1678.

Yu AS. 2003. Claudins and epithelial paracellular transport: the end of the beginning. Curr Opin Nephrol Hypertens. 12(5):503-509.

C h a p t e r 3 Gene Expression by 18-day Embryonic Chick Intestine in the Absence and Presence of Dexamethasone

ABSTRACT

Glucocorticoids are essential for the maturation of numerous tissues including the intestine during embryonic development. They have also stimulatory effects on the integrity of tight junctions. In this study, if a synthetic glucocorticoid, dexamethasone has any effect on mRNA expression of tight junctional proteins was investigated in 18-day embryonic chick intestine *in vitro* using microarrays and quantitative real time PCR analyses. Real-time PCR analyses revealed that both claudin-3 and claudin-5 tight junction gene expressions were induced in response to DEX in a dose dependent manner.

The objectives and significance of research:

Our aim was to investigate the effects of glucocorticoids (GCs) on the expression of tight junctional (TJ) and some additional regulatory genes related to TJs in organ-cultured embryonic chick intestine using microarray analyses. Pre-hatched chick intestine will provide us a convenient model because the organization of the barrier is not completely established, and it is relatively easy to maintain its morphological integrity for at least 24 hours in an organ culture system.

The stimulative effects of GCs on the barrier function of TJs in the mammary gland, intestine, lung, kidney and cerebral endothelia were demonstrated using transepithelial resistance (TER) and paracellular permeability studies *in vitro* and/or *in vivo* (Burek and

Forster, 2008; Boivin et al., 2007; Peixoto and Collares-Buzato, 2006; Romero et al., 2003). Moreover, enhancing effects of GCs on the expression of endothelial occludin, ZO-1 and claudin-5 (cldn-5) were reported (Burek and Forster, 2008; Forster et al., 2008; Forster et al., 2005; Stelwagen et al., 1999). However, not much is known about the effects of GCs on the expression of other tight junctional proteins in various epithelia, even though GCs are currently used clinically for the treatment of various epithelial permeability dysfunctions. In this project, we utilized microarrays to investigate the changes in the expression of 385 genes were investigated in 18-day pre-hatch chick intestine in response to 1μM dexamethasone (DEX, a synthetic GC) after 12 hours in vitro. In addition, the expression of cldn-3 and cldn-5 were further analyzed in response to different concentrations of DEX at different time points. This project will provide us valuable preliminary data for future studies on GC-mediated regulation of TJs.

LITERATURE REVIEW

Small intestine is a good model organ to study the expression and functions of claudins because a variety of physiological events, such as absorption, final enzymatic digestion of macromolecules, secretion, proliferation, cell migration, apoptosis and acid-base balance occur within the luminal epithelium. The mucosal surface of the intestine is covered by a single epithelial cell layer. The epithelium is arranged to maximize the surface area at several levels. After forming rough folding, it is organized into finger-like structures so-called villi. Each villus can be considered a functional unit of the small intestine. The bottom regions of villi are called “crypt regions”. These regions include multi-potent stem cells. Stem cells proliferate by mitosis, and newly formed cells migrate up to the villus tip. During the migration period they differentiate into one of three main different cell types: absorptive cells, enteroendocrine cells, and goblet cells (Crosnier et al., 2006; Madara and Trier, 1994).

In chick embryos, rapid functional differentiation of the epithelium occurs between days 14 and 21 (the week before hatching), including changes in cell shape, formation of microvilli and the terminal web, and a dramatic increase in the density of goblet cells (Black, 1978; Black and Smith, 1989; Uni et al., 2003). The organization and maturation of epithelial and endothelial TJs occur during this period as well. Claudin protein family is the main component of TJ strands and these proteins are directly involved in the barrier function of TJs (Sonoda et al., 1999). Additionally, the claudin protein family with its more than 20 members creates charge and size selective pores in the paracellular pathways, thus

exerting a critical influence in the composition of the transported solutes in a tissue specific manner (Van Itallie and Anderson, 2006; Colegio *et al*., 2002; Rahner *et al*., 2001).

The Effects of Glucocorticoids (GCs) on TJs and Claudins:

GCs are one class of steroid hormones, produced by the adrenal cortex. Their secretion is regulated by adrenocorticotropic hormone (ACTH) from the anterior pituitary. GCs are bound to a carrier protein called corticosteroid-binding globulin during their circulation in the blood (Buckingham, 2006).

GCs bind to a cytoplasmic glucocorticoid receptor (GR) in the target cell. This receptor-ligand complex translocates into the cell nucleus and binds specific DNA sequences called glucocorticoid response elements (GRE) in the promoter regions of a specific gene; and consequently, changes gene transcription (Beato, 1989).

GCs are crucial for differentiation and maturation of various tissues during embryonic development in mammals and birds (De Groef et al., 2008; Wada, 2008; Bossis et al., 2004; Jenkins and Porter, 2004; Fisher 1992; Black, 1988a; Black 1988b; Black and Moog, 1977). The primary GC in birds is corticosterone at the last stages of embryonic development and after post-hatch; however, in the earlier stages, both corticosterone and cortisol were detected at approximately equal amounts (Kallecharan and Hall, 1974; Nakamura et al., 1978). GCs are detected in small amounts in the adrenal glands by day 8; and later in the plasma ($\sim 10^{-2}\mu M$) by day 10 of embryonic development (Kalliecharan and Hall, 1974; Wise and Frye, 1973; Pedemera, 1972). During 14-16 days of development, there is an increase in serum corticosterone and cortisol concentration ($\sim 2.10^{-2}\mu M$)

probably due to initiation of ACTH secretion during this period (Jenkins and Porter, 2004). The day before hatching, the second surge of GCs is seen, reaching a level of ~3.10^{-2}μM in the serum. Levels drop slightly after hatching (Wise and Frye, 1973; Scott et al., 1981; Marie, 1981). Gradual increase in GC concentrations between day 17 and 20 has been suggested to play an important role in physiological changes in intestinal (duodenal) epithelia such as differentiation, and increase in the activity of alkaline phosphatase and maltase in chick (Black and Moog, 1977 and 1978; Moog and Ford, 1957; Moog and Richardson, 1955). GCs induce the activity of active glucose transport *in vitro* (Black, 1988a).

In addition to developmental roles, GCs have metabolic, immunologic and various other physiological roles in almost every tissue in the body. In the fasted state, GCs increase blood glucose levels by stimulating gluconeogenesis, glycogenolysis, lipolysis and aminoacid mobilization in mammals. They also have well-known immuno-suppressive and anti-inflammatory activities. They regulate cell differentiation and growth, proliferation, apoptosis, cell survival, cell cycle, signal transduction, neurotransmission, and epithelia and endothelia permeability (Buckingham, 2006; Felinski and Antonetti, 2005).

GCs induce the expression and/or reorganization of TJ proteins in the epithelial and endothelial cell layers; and accordingly, they promote the tightening of the TJ barrier (Felinski and Antonetti, 2005). Abnormalities in the barrier function of TJs (increase in paracellular permeability) are seen in various pathological conditions, such as in Crohn's disease, alcoholic liver disease, celiac disease and various diarrheal syndromes (Ma and

Anderson, 2006). GCs are one of the therapeutic reagents that are used for the treatment of Crohn's disease by re-tightening of TJs (Wild et al., 2003; Rutgeerts, 2001a and b). A number of studies have showed that GC treatment induce a tightening effect on cerebral endothelial TJs in vitro (Romero et al., 2003; Hoheisel et al., 1998; Forster et al., 2005; Weksler et al., 2005). In endothelial cells, GC treatment changes the position of occludin by its dephosphorylation, and further increases the expression of other TJ proteins to form tighter TJs (Antoletti et al., 2002). In cultured brain endothelial cells, 1μM DEX treatment decreased paracellular permeability for sucrose, flurescein and dextrans of up to 20KDa, probably modulating the expression and/or organization of TJ proteins (Romero et al., 2003). GCs were suggested to support the integrity of blood-brain barrier (BBB), and they are currently used for the treatment of brain tumors. GCs are also considered a potential treatment for diabetic retinopathy, which is characterized by the loss of blood-retinal barrier (BRB) (Felinski and Antonetti, 2005; Rutgeerts, 2001a). In addition to their tightening effects on endothelial TJs, GCs also affect the strength of epithelial layers in various tissues.

DEX treatment of 31EG4 mammary epithelial cells showed a 65-fold increase in transepithelial electrical resistance (TER is a measure of tightness of TJs) and a 10-fold decrease in paracellular radiolabeled mannitol flux. GCs were reported to increase the cellular expression of occludin and ZO-1 TJ proteins in mammary cell lines (Stelwagen et al, 1999; Buse et al., 1995; Singer et al., 1994; Zettl et al, 1992).

An actin bundling protein, fascin was proposed to be a mediator whose downregulation results in induction of TJ formation and TER in rat mammary epithelial tumor cells (Wong et al., 1999). GCs decreased the levels of fascin and eliminated the inhibitory effects of fascin on the formation of TJs. The effects of GCs on fascin activity were proposed to be blocked by TGFα (Guan et al., 2002).

GCs are claimed to decrease TJ permeability significantly (shown by decreased paracellular radiolabeled inulin flux) by suppressing myosin light chain kinase (MLCK) gene activity in Caco-2 intestinal cell lines (Boivin et al., 2006).

Exposure of subconfluent Madin-Darby Canine Kidney Cells (MDCK) renal epithelial cells, in which the formation of epithelial barrier is not complete, to 4 μM DEX for 24h did not affect the expression of cldn-1, ZO-1, and occludin, but it significantly increased the TER, probably by modulating the organization and relocalization of TJ proteins to the TJ areas (Peixoto and Collares-Buzato, 2006). In these studies, DEX had a stronger effect on the formation of the epithelial barrier in subconfluent epithelial cells than in the confluent cells with relatively stable TJ structure (Peixoto and Collares-Buzato, 2006). Chronic exposure to 4μM DEX exposure for 72 hours also increased TER significantly in confluent MDCK monolayers (Peixoto and Collares-Buzato, 2006).

Hypotheses:

Current literature supports the correlation of tighter TJs in various tissues with the effects of GCs. The effects of GCs on TJs could be explained by two ways: first, GCs would directly change the expression and/or activity of TJ proteins, specifically claudins.

Secondly, they might influence the expression and/or activity of other proteins that have regulatory functions on the organizations of tight junctional proteins. While some claudin proteins form the barrier function of TJs by sealing the paracellular spaces, some of them, like cldn-2, form pore-like structures in the paracellular pathways and make them leakier. Therefore, DEX would increase certain claudins which play roles in forming the barrier, but might down-regulate those claudins which form pore-like structures in the paracellular areas.

MATERIALS and METHODS

Animals:

Fertile broiler-type chicken fertile eggs were obtained from the Poultry Farm of North Carolina State University and incubated for 18 days in a humidified incubator at 99-100^{o}F.

Tissue Preparation for Staining:

Embryonic chicks were sacrificed, and the duodenal loop was immediately removed and rinsed with cold phosphate buffered saline (PBS), pH 7.4 to remove blood and debris. Tissue approximately 2-3mm long pieces were taken from the duodenum loop, split opened and placed into PBS.

Intestinal Organ Culture:

Three 25-ml Erlenmeyer flasks including 3ml culture medium (Medium 199, GIBCO) and streptomycin were prepared and supplemented with a synthetic

glucocorticoid, (DEX) with final concentrations of 0μM, 10^{-2}μM, 10^{-1}μM, and 1μM (Figure 3.2). Duodenal segments were placed into flasks and gassed with a mixture of 95% O_2 and 5% CO_2. Flasks including tissue pieces were incubated in a 37ºC incubator. Cultured tissue pieces were preserved in RNA*later* (Ambion) solution and stored at -20ºC.

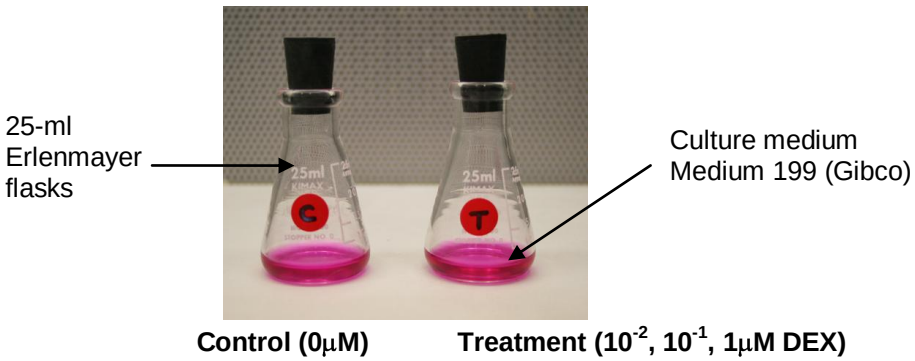


Figure 3.1: Organ culture. Intestinal organ culture was performed in 25-ml Erlenmeyer flasks containing "Medium 199".

Isolation of Epithelial Cells from Intestinal Villi:

Isolation of epithelial cells from intestinal villi was performed as described by Black (1988a). Briefly, tissue pieces removed from the small intestine were incubated in citrate buffer for 20 minutes at room temperature. Then, they were transferred to an EDTA-containing buffer and incubated at 37^0C with shaking for 30 minutes. The resulting cell suspension was filtered through silk screen to remove tissue debris and centrifuged for 5 minutes to obtain a pellet of epithelial cells. The cells were re-suspended in PBS buffer and utilized for real-time PCR analyses.

Total RNA Extraction:

Organ cultured tissues were homogenized in RLT buffer (Qiagen) including β-mercaptoethanol using a bead beater. Homogenate was incubated at room temperature for 10 minutes followed by the addition of phenol/chloroform/isoamyl alcohol mix (125:24:1). The mixture was vortexed and centrifuged. The upper aqueous phase contained total RNA. The concentration and purity of the total RNA samples were measured by a Nanodrop spectrophotometer. Total RNA with sufficient purity ($A_{260/280}$>1.8) were used for further analyses. The quality of the total RNA was further analyzed by 1.5% agarose gel. Total RNA was purified with DNase according to manufacturer instructions (Ambion).

Microarray Analyses:

Microarray analysis was performed to detect genes whose expressions were significantly changed in response to DEX exposure with different concentrations and exposure times. 70-mer oligonucleotides were ordered from Operon Biotechnologies, Inc. Oligos were placed in a 384 well plate with 100pmol/μl concentrations. Then, they were mechanically printed in specific locations on amino-silane coated glass slides (Corning UltraGAPS Slides) using a robotic BioRad printer (contact printing method). Each oligo was printed twelve times at different locations of the slides as technical replicates. Each array includes 384 unique oligos for 384 genes. 98 of these genes are involved in tight junctions, cell cycle, apoptosis, metabolism, immunologically related and cell signaling related genes (Table 3.1). The rest of the genes are mostly related to metabolism.

Table 3.1: List of tight junction related genes in the arrays.

Claudin	Cell adhesion	Signaling genes	Cell cycle	Apoptosis	GF	Immunological molecules	other
Claudin-1	ZO-1	β catenin	cdc42	Bcl-2	FGF	IFNAR2	Na_K ATPase
Claudin-2	ZO-3	c-Myc	CDK1	14-3-3	FGFR	IFNGR2	CD36
Claudin-3	MLCK	EGF	CDK2	FasL	EGF	IL10	MMP2
Claudin-4	E-Cadherin	ERK1	cyclinD1		VEGF_A	IL10RA	MAG1
Claudin-5	Activin-A	ERK2	E2F		VEGF_R2	IL11RA	PP2A
Claudin-10	Activin-AR	F-actin	p21Cip1			IL12 receptor β2	
Claudin-11	Activin-R	Fascin	p27Kip1			IL13	
Claudin-12	Occludin	Gluco. Rec.				IL13RA2	
Claudin-13	ICAM 1	HDAC				IL15	
Claudin-19	JAM	HNF4a				IL17R	
Claudin-22	NCAM	Id_1				IL1β	
Claudin-24	Cingulin	JNK1				IL1R1	
	Selectin	JNK2				IL-2 precursor	
		MAP3K2				IL2RA	
		MEK				IL3	
		p38 MAPK				IL5	
		p53				IL7	
		PAR3				IL-8	
		PI3K				Interferon alpha-A pre.	
		RAB1A				Interferon gamma pre.	
		Rac1				Interferon type B pre.	
		RhoA				IRF10	
		Smad2					
		Smad5					
		TGF-α					
		TGF-α R					
		TGF-β1					
		TGF-β1 R					
		TGF-β2					
		TNF Rec.					
		AP1					

RNA samples from each treatment were reverse transcribed to cDNA by ChipShot indirect labeling kit (Promega), and then these cDNA groups were labeled with Cy3 and Cy5 fluorescence dyes (GE Healthcare). cDNAs labeled with two different dyes were hybridized with the same microarray slide (Pronto! Validation Kit, Corning). The arrays were scanned by a dual-laser DNA microarray scanner, and processed by the ScanAnalize Software (Stanford University). Statistical data analyses were done using JMP Genomics 3.0 Software. Briefly, data were converted to $\log_2$ transformation and then normalized

based on a global adjustment to reduce technical variation with the following equation: $[\log_2(I_j) - m]/s$ where I_j= intensity of the j^{th} spot, m=mean or median, s=standard deviation. Genes whose expressions were significantly altered were evaluated by ANOVA with false discovery rate.

Further analyses for the optimal time and concentration effect of DEX on the expressions of these genes were performed by real-time PCR technique.

SYBR Green Real-Time PCR Analyses:

Reverse transcription and SYBR green real-time PCR analyses were performed as described in Chapter 2. 18S, β-actin and GAPDH housekeeping genes were tested if they were stable in different experimental conditions or during the experiment, and β-actin was decided to use as an internal control. Cycle threshold (Ct) values were obtained using the Icycler Software, duplicate values of each cDNA were averaged, and relative fold changes were determined using $2^{-\Delta\Delta Ct}$ method. Experimental errors were calculated by the standard error of the mean (SEM) of normalized Ct values from each treatment group, and were indicated as error bars in the graphs. Least Square Means differences with Student's t test were performed to determine the significance between treatment groups. P value < 0.05 was accepted as statistical significance. The annealing temperatures and the expected sizes of produced amplicons for cldns, and the real-time PCR protocol are shown in Table 1 and 2 in Chapter 2, respectively.

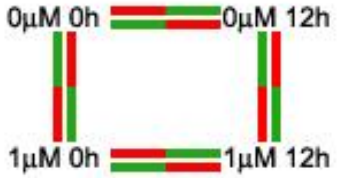
Experimental Designs:

Experimental design #1 - Microarray analysis: 18-day pre-hatch chick intestine in 1μM DEX supplemented medium (Medium 199, Gibco) was cultured for 12 hours and screened the changes in gene expression by comparing the control group which did not include any DEX (Fig. 3.3).

Treatments	**Time (hr)**	
Control culture (0μM)	0	12
DEX exposure (1μM)	0	12

Figure 3.2: DEX exposure for microarrays. Concentration and exposure times of DEX for microarrays analyses.

Total RNA was isolated from four treatment groups (explained below). After DNase treatment, RNA was converted to cDNA by using random primers and oligo dT primers. When the cDNA concentration was high enough (>20ng/μl) and it had sufficient purity (A280/260>1.8), cDNAs from each treatment group was labeled with two times Cy3 and two times Cy5 dyes. The assignment of the dyes and the comparison of the treatment groups were summarized below (Fig. 3.4). Each array had a replicate labeled with the reverse dye; and accordingly, 16 labeling reactions were performed and 8 arrays (8 hybridizations) were formed.



4 comparisons x 2 (dye flipping) =
8 arrays x 2 (Cy3, Cy5) =
Total: 16 labeling reactions.

Figure 3.3: The assignment of the dyes and the comparison of the treatment groups.

Experimental design #2: SYBR green real-time PCR analyses of cldn-3 and cldn-5 in response to different concentrations of DEX at different exposure times: four different concentrations of DEX were used: control (0μM), close to physiological level (10^{-2}μM), moderate level (10^{-1}μM), and high dose (1μM) of DEX. Samples were taken from time 0, 12, and 24 hours of cultures (summarized in Fig. 3.5).

Treatments:	**Exposure times (hr)**		
0μM DEX	0	12	24
10^{-2}μM DEX	0	12	24
10^{-1}μM DEX	0	12	24
1μM DEX	0	12	24

Figure 3.4: Concentration and exposure times of DEX for QPCR analyses.

RESULTS

A volcano plot is an efficient way to summarize and present both the fold change and the statistical significance of a gene at the same time (Fig. 3.6). Each dot in the volcano plot represents a gene. Since there are 385 features, there are 385 dots on the plot. The *X*

axis gives us information about fold changes of a gene, while the right hand side of the 0.0 value indicates up-regulation and the left side indicates down-regulation of a gene. The *Y* axis gives us information about the statistical significance for the change of a given gene expression. The higher the point, the more statistically significant the fold changes of a given gene.

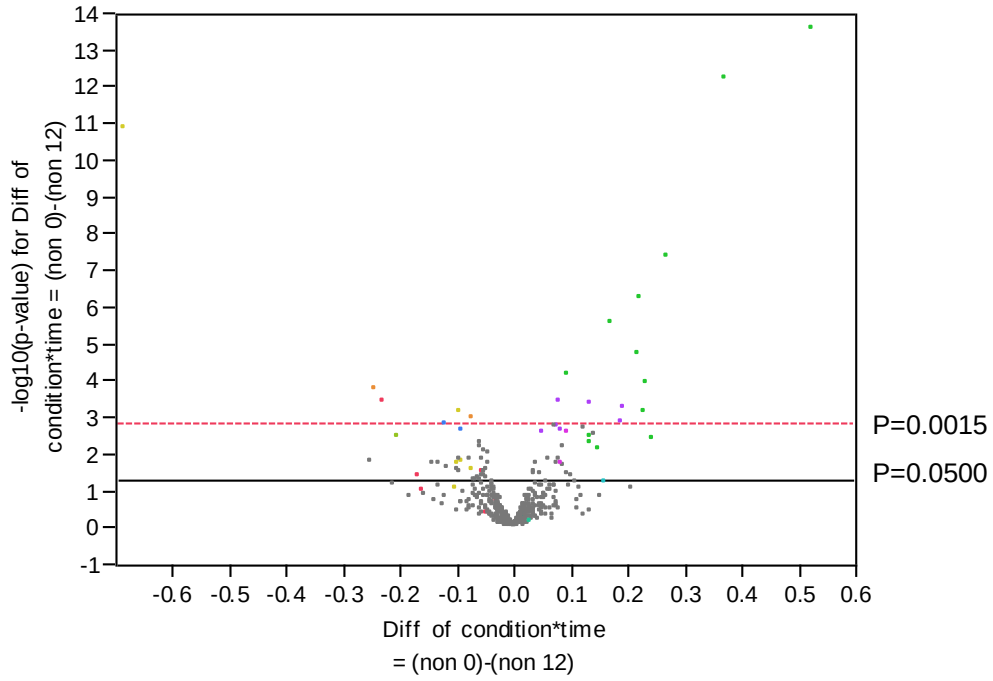


Figure 3.5: Visualization of microarray data using a volcano plot.

Changes in gene expression in the culture condition over 12 hours (time effect):

The changes in the expression of 385 genes were determined in 18 days old pre-hatch chick intestine incubated in the control culture for 12 hours *in vitro*. Numerous genes were significantly up-regulated, and relatively smaller numbers of genes were down-regulated. Genes whose expressions are up-regulated include smooth muscle tissue related genes, extracellular matrix components, Krebs cycle, cell cycle, cell signaling, growth, and

cell differentiation related molecules. No claudin gene up-regulation was detected. The complete list of the genes whose expression levels were up-regulated is shown in Table 3.2.

Table 3.2: List of genes whose expression levels were up-regulated during 12 hours of culture. The order of genes is organized according to their p values.

Gene	ID No	Explanation	-log10(p value)	Probability (P value)	Fold change
SM22	TC189636	Smooth muscle cell spe. prot.	13.525	2.98538E-14	1.43654
MYL9	TC210862	Myosin light polypeptide 9 regu.	12.194	6.39735E-13	1.2945
DCN	TC209608	Decorin	7.326283126	4.71755E-08	1.20489
FSTL1	395349	Follistatin-like 1	6.227730157	5.91929E-07	1.16586
14-3-3	TC229596	14-3-3	5.532150613	2.93663E-06	1.12405
ENO1	396017	Enolase 1	4.675884858	2.10919E-05	1.16264
F-actin	TC228202	F-actin	4.104296915	7.86508E-05	1.06814
ActivinR	TC228579	Activin receptor I pre.	3.408901842	0.00039003	1.05709
ERK2	TC230693	Extracell. signal-reg. kinase 2	3.317196459	0.00048173	1.09825
ALDH2	TC208477	Aldehyde dehydrogenase	3.214315097	0.000610499	1.14163
SPARC	TC187113	Secr. protein acidic cysteine-rich	3.123227157	0.000752962	1.17129
IDH2	TC210085	isocitrate dehyd. (NADP+)	2.842092959	0.001438491	1.14092
MAP3K2	TC275325	MAP3K2 protein	2.714367616	0.001930334	1.05241
SUCLG1	TC208213	Succinate CoA ligase	2.704104699	0.001976493	1.05468
Gal-1 α	TC279522	Gallinacin-1 α pre.	2.653369955	0.002221417	1.08922
CDK1	TC230512	Cyclin-dependent kinase 1	2.596692019	0.002531092	1.05981
CyclinD1	TC236886	G1/S-specific cyclin-D1	2.575013099	0.002660645	1.03433
HSPE1	TC196302	Heat shock 10kDa protein 1	2.52670452	0.002973689	1.06807
RAB1A	TC249692	RAB1A, memb. RAS onco. fam.	2.482977013	0.00328869	1.10359
ELN	TC193337	Elastin	2.42490951	0.003759157	1.09612
HOXA3	395231	Homeobox A3	2.288652373	0.005144553	1.09817
INSR	TC210106	Insulin receptor	2.133787158	0.007348739	1.06054
GAPDH	TC207111	glycerald. 3-phosphate dehydro.	2.124494395	0.007507677	1.10711
JNK2	TC229898	Mitogen-activated prot. Kin. 9	1.81262921	0.015394684	1.04406
ERK1	TC298015	MAPK/ERK kinase kinase 1	1.720951918	0.019012888	1.02733
MYL4	396472	Myosin light polyp. 4 alkali; atria	1.692093167	0.020319211	1.05497
SDHB	TC225543	Succinate dehyd. Ip subunit	1.692093167	0.020319211	1.05497
p53	TC229905	Cell. tumor antig. p53 (Tum. sup	1.692055503	0.020320973	1.0601
CKM	TC186914	creatine kinase	1.66495472	0.02162944	1.06231
smad-2	TC265847	Smad2	1.401736825	0.039651824	1.02576
MMP2	TC228355	Matrix metalloproteinase-2	1.151710382	0.070516317	1.02719
Cldn3	TC229685	Chick claudin3	0.995810104	0.100969428	1.04232

In 18-day embryonic chick intestine, some cell signaling molecules, growth factors, and carbohydrate metabolism related genes were significantly down-regulated even though the fold changes were less than 15%. Cldn-10 and cldn-2 were very slightly but

significantly down-regulated. The complete list of the genes whose expression levels were down-regulated is shown in Table 3.3.

Table 3.3: List of genes whose expression levels were down-regulated during 12 hours of culture. The order of genes is organized according to their p values.

Gene	ID No	Explanation	-log10(p value)	Probability (Pvalue)	Fold change
HB alpha/	TC207660	Hemoglobin alpha-A	3.713551561	0.000193396	0.84428
HYOU1	TC194969	Hypoxia up-regulated 1	3.378538448	0.000418275	0.85352
SLC2A1	TC191959	Solute carrier family 2	3.134553742	0.000733578	0.93504
KCNA3	404303	Potass. Volt -gated chan. Shake	2.948422517	0.001126101	0.9504
GBE1	TC191346	glycogen branching enzyme	2.7691248	0.001701669	0.91923
IL2	TC214019	Interleukin-2	2.611821231	0.002444437	0.9392
HBB	TC207666	Hemoglobin beta	2.4602176	0.003465632	0.8679
CXCR4	TC230261	CXCR4	2.261309228	0.005478867	0.96033
Cldn10	TC290376	Claudin-10	2.038784231	0.009145675	0.96433
TGF_b2	TC230416	Transf. growth factor b-2 pre.	2.002039163	0.009953157	0.96916
Rho_A	TC232117	RhoA GTPase	1.794025421	0.016068472	0.96133
ZC3H15	TC224514	Erythropoietin 4 immediate early	1.7480513	0.017862766	0.93874
Cldn2	TC277831	Claudin2	1.703383855	0.019797764	0.9705
PYGL	TC225625	glycogen phosphorylase	1.680838281	0.020852672	0.91345
VEGF_A	TC246964	Vascular endothelial growth fact.	1.595086674	0.025404656	0.9214
Bcl_2	TC250722	Apoptosis regulator Bcl-X	1.558189845	0.027657324	0.96754
GHRHR	TC221240	Growth horm. releasing horm. R	1.519650648	0.03022382	0.93329
ANF / ANI	TC208792	Atrial natriuretic factor pre.	1.516427835	0.030448939	0.95086
FLK1	38649422	Vascular endoth. growth fact. re	1.501640173	0.031503574	0.9652
DIO2	373903	Deiodinase iodothyronine type II	1.490894787	0.032292764	0.96299

Changes in gene expression in response to DEX *in vitro* over 12 hours (DEX effect): Fewer numbers of genes were up- and down regulated in response to DEX relative to the changes in "time effect" (Table 3.4). Interleukin-2 (IL-2), glutamine synthetase, glycogen phosphorylase, and glycogen branching enzyme were significantly up-regulated even though the fold changes were not more than 22%. On the other hand, Fas ligand, hypoxia up-regulated 1, cldn-3, deiodinase iodothyronine type II, and hemoglobin beta were significantly but very slightly down-regulated (less than 11%).

Table 3.4: List of genes whose expression levels were down- (blue) and up-regulated (pink) in response to DEX during 12 hours of culture:

Gene	ID No	Explanation	-log10(p value)	Probability (P value)	Fold change	up/down
IL2	TC214019	Interleukin-2	3.073464289	0.00084438	1.06925	up
GLUL	TC210122	Glutamine synthetase	2.44552835	0.00358486	1.22706	up
PYGL	TC209295	Glycogen phosphorylase	2.180003675	0.00660688	1.04935	up
GBE1	TC191346	Glycogen branching enzyme	1.973893924	0.01061955	1.06829	up
IRF2	TC210368	Interferon regulatory factor 2	1.922622941	0.01195025	1.20072	up
CCL2	TC289521	Small inducible cytokine A2	1.679468797	0.02091863	1.04131	up
STAT2	TC299780	Signal transducer and activ. of trar	1.538738694	0.0289242	1.045	up
IL2RA	TC230927	IL-2 receptor alpha subunit	1.138024271	0.07277391	1.09612	up
Cldn13	TC268889	Claudin13	1.028355206	0.09367955	1.03699	up
NKX2-5	396073	NK2 transcription factor related loc	0.974965915	0.10593369	1.05981	up
IL13	TC221524	Interleukin-13	0.944029096	0.11375511	1.06873	up
VEGF-A	TC246964	Vascular endothelial growth factor	0.942627323	0.11412287	1.05797	up
p53	TC229905	Tumor suppressor p53	0.919401179	0.12039233	1.03922	up
Cldn4	TC299414	Claudin4	0.649504343	0.22412776	1.016	up
Cldn11	TC294790	Claudin11	0.565420182	0.27200684	1.01685	up
Cldn2	TC277831	Claudin2	0.539040292	0.28904117	1.01305	up

Gene	ID No	Explanation	-log10(p value)	Probability (P value)	Fold change	up/down
FasL	TC234344	Fas ligand	2.956136794	0.00110628	0.94377	down
HYOU1	TC194969	Hypoxia up-regulated 1	2.363676562	0.00432836	0.88964	down
Cldn3	TC229685	Claudin3	2.017241913	0.00961077	0.93803	down
DIO2	373903	Deiodinase iodothyronine type II	1.728158634	0.01869999	0.95926	down
HBB	TC207666	Hemoglobin beta	1.443451499	0.0360204	0.90897	down
Cldn5	TC256697	Claudin5	0.189294959	0.64670325	0.99165	down
Cldn1	TC228768	Claudin1	0.096637012	0.80050304	0.99426	down

Real-time PCR analyses of claudin-3 and -5 expression in response to DEX:

The expression of cldn-3 and -5 was further investigated using comparative SYBR Green Real-Time PCR analyses.

Cldn-3 expression was increased in a dose dependent manner in response to DEX (Fig. 3.7A). There was no significant time effect on cldn-3 in a given DEX dosage between 12h and 24h of exposures. At physiological levels ($10^{-2}\mu M$) DEX did not induce any

significant increase in cldn-3 for 12h. Moderate ($10^{-1}\mu M$) and high levels ($1\mu M$) of DEX exposure for 12h caused approximately 2.5 and 3.2 folds increase in cldn-3 expression, respectively (Fig. 3.7B). DEX exposure for 24 hours caused a dose dependent increase in cldn-3 mRNA levels; however none of them were significant (Fig. 3.7C).

Relative cldn-5 mRNA levels in response to different concentrations of DEX at different time points were also investigated (Fig. 3.8). Cldn-5 displayed a similar pattern to cldn-3 expression. At a concentration of $10^{-2}\mu M$, DEX did not induce any significant increase in cldn-5 for 12h exposure (Fig. 3.8A). However, moderate ($10^{-1}\mu M$) and high ($1\mu M$) doses of DEX exposure for 12h induced cldn5 by approximately 3.4 and 5 fold, respectively (Fig. 3.8B). 24 hours of DEX exposure caused a dose dependent increase in cldn-5 mRNA levels; however none of them were significant (Fig. 3.8C).

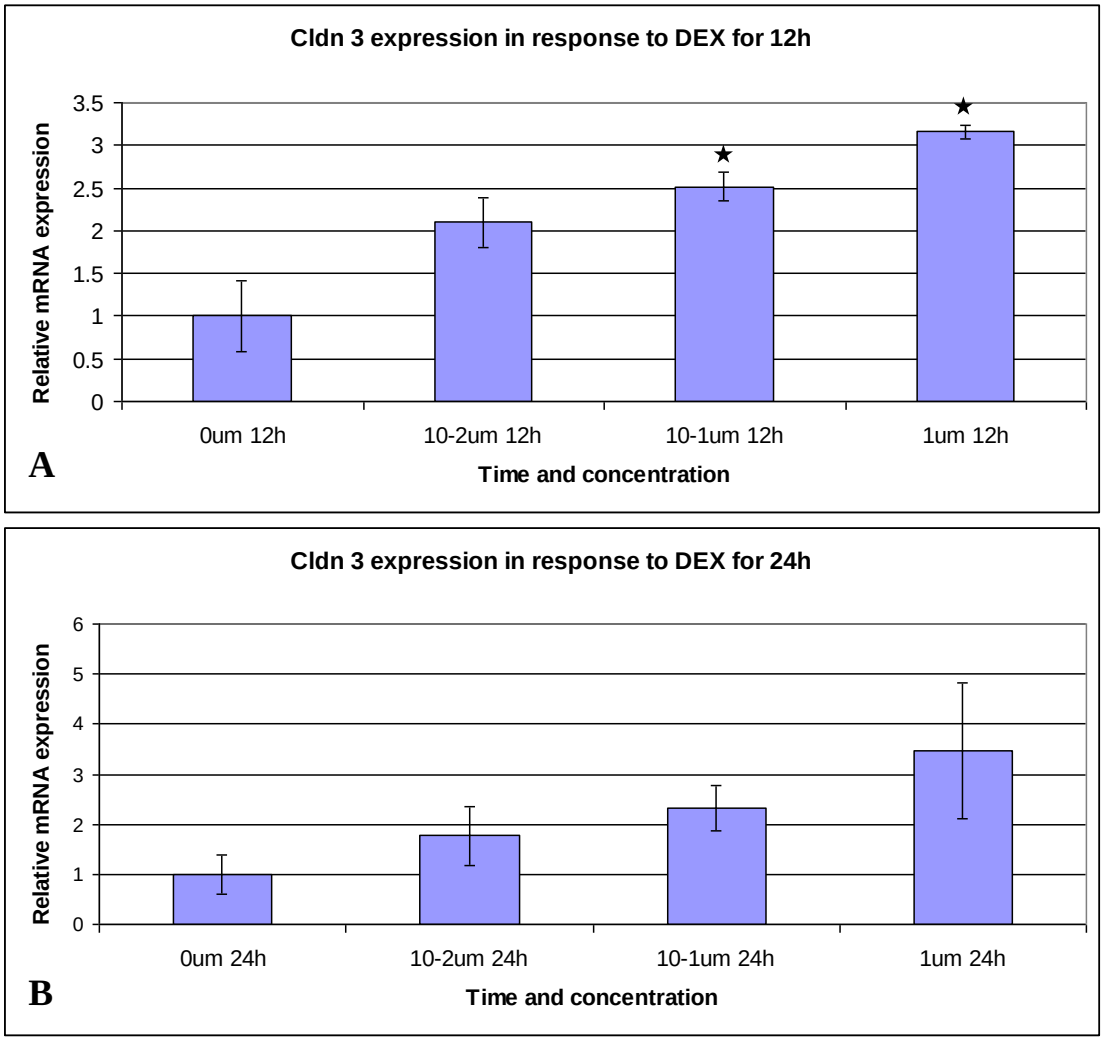


Figure 3.6: Relative expression of cldn-3 in response to DEX. The effects of different concentrations of DEX on cldn 3 expression for 12h (A), and for 24h (B). n= 3-4. "★" indicates $p < 0.05$.

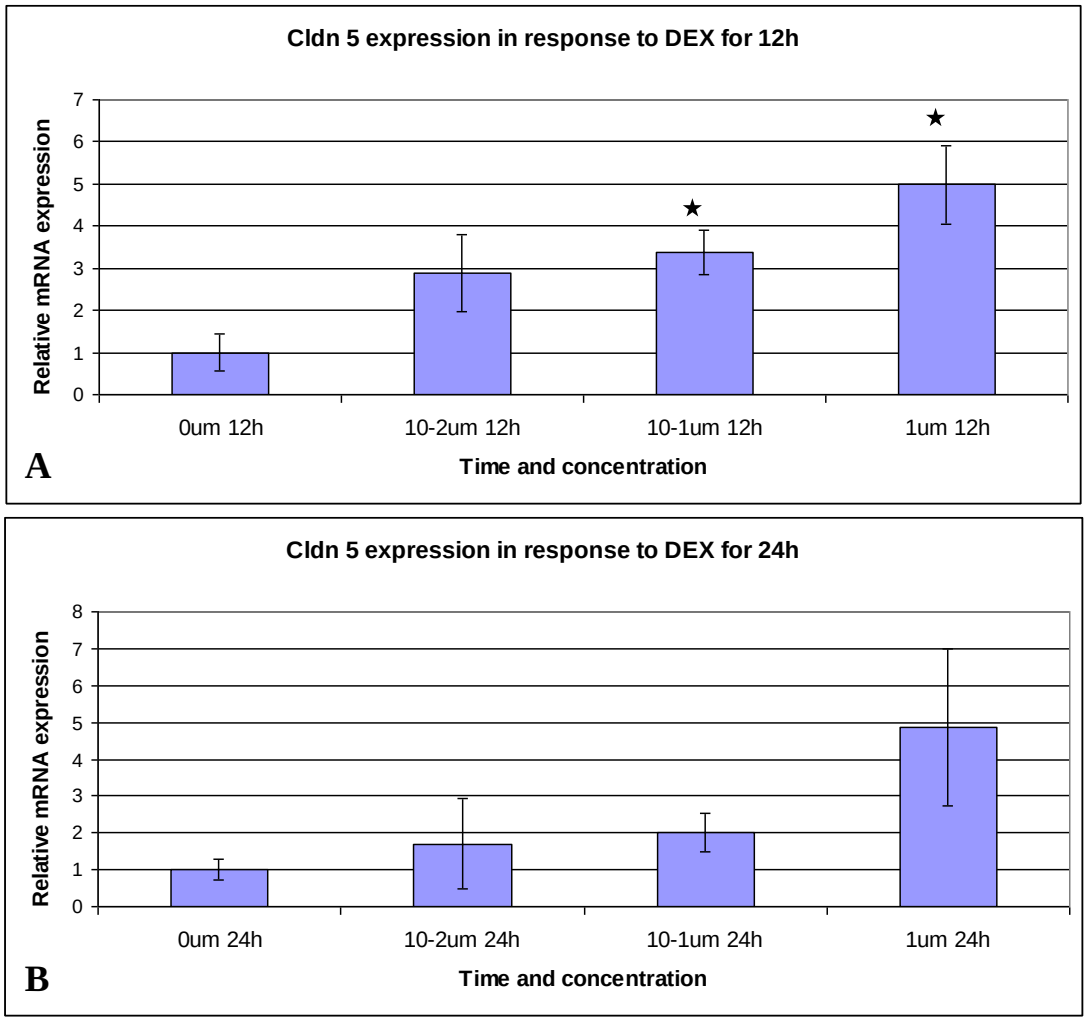


Figure 3.7: Relative expression of cldn-5 in response to DEX. The effects of different concentrations of DEX on cldn-3 expression for 12h (A), and for 24h (B). n= 3-4. "★" indicates $p < 0.05$.

DISCUSSION

GCs have wide range of effects in the body and modulate approximately 10% of human genes. GCs control metabolism, immune system, growth, differentiation, and development (Buckingham, 2006). GCs also play essential roles for growth differentiation,

metabolism and morphogenesis of numerous mammalian and avian embryonic tissues and organs such as the intestine, lung and central nervous system (Buckingham, 2006). GCs play important roles in functional differentiation of intestinal epithelium during the last week of chick embryonic development (Black, 1988a; Black 1978; Moog and Richardson, 1955). In addition, usage of exogenous GCs is important for the treatment of various pathological conditions due to anti-inflammatory and epithelial and endothelial barrier enhancing qualities of GCs.

Disruption of epithelial and endothelial paracellular barriers in various tissues is associated with pathological conditions such as edemas in various tissues, mucosal inflammation, and damage to the BBB (Jepson et al., 1995; Rubbin and Staddon, 1999; Sonoda et al., 1999). Abnormalities in the permeability of paracellular pathways in the intestine precede the onset of Crohn's and celiac diseases (Hollander, 1988; Irvine and Marshall, 2000). Changes in the expression and organization of occludin, cldn-2, -3, -5, and -8 proteins were reported in Crohn's disease (Zeissig et al., 2007). The negatively regulating activities of proinflammatory cytokines on TJs and endocytosis of TJ proteins are suggested to bring about weakened barriers (Laukoetter et al., 2006). GCs are currently used therapeutically to increase the integrity of epithelial barrier function (Irving et al., 2007). Glucocorticoid exposure to epithelial and endothelial layers reduces paracellular permeability. GCs reduce edema by decreasing capillary permeability in vivo (Guyton and Hall, 1996). Consistently, enhancing effects of GCs on the formation and maintenance of TJs have been reported for endothelial and epithelial cells in vitro (Romero et al., 2003; Stelwagen et al., 1999; Zettl et al., 1992).

Assembly and disassembly of TJ proteins is dynamic and controlled by numerous signals, transcription factors and metabolic regulators such as second messengers, snail, slug, β-catenin/Tcf, T/EBN/NKX2.1 at different developmental stages and physiological conditions (Ikenouchi et al., 2003; Miwa et al., 2001; Niimi et al., 2001; Walsh et al., 2000; Buse et al., 1995; Anderson et al., 1993; Madara, 1988). For example, TJ barrier is down-regulated at gene and post-transcriptional levels by inflammatory cytokines such as tumor necrosis factor (TNFα), interferon-gamma, and interleukin 13 (Walsh et al., 2000; Prasad et al., 2005; Tedelind et al., 2003).

GCs affect cldn-5 expression directly and indirectly in endothelial cell lines (Burek and Forster, 2008). Tumor necrosis factor-alpha (TNFα) down-regulates the expression of cldn-5 in brain cEND and myocardial MyEND endothelial cells (Burek and Forster, 2008). DEX indirectly up-regulates cldn-5 expression by down-regulating the expression of TNFα in these endothelial cell lines (Burek and Forster, 2008). Additionally, DEX can induce both the promoter activity and mRNA levels of cldn-5 directly in cEND cells but not in MyEND cell lines, which indicate the tissue specific actions of GCs. In this study, as it was hypothesized, GCs induced the expression of cldn-3 and -5 in a dose dependent manner. However, it is not known if DEX directly regulated the expression of these genes by binding their GRE regions in their promoters or indirectly changed the activity of other genes such as TNFα, cytokines, and glutamine synthetase.

In our microarrays analyses, glutamine synthetase (GS) gene was up-regulated. GS is an important enzyme for maintaining physiological levels of glutamine. This enzyme has

been reported to be important for intestinal cell differentiation shown in Caco-2 cell line (Weiss et al., 1999). Glutamine is crucial for intestinal mucosal integrity, metabolism and function (Neu et al., 2002; Dugan and Mcburney, 1995). It serves as an energy source and provides precursors for nucleotide biosynthesis (Bulus et al., 1989; Smith, 1990). It has been suggested that it is important for cell proliferation in cultured rat intestinal crypt cells (DeMarco et al., 1999). Glutamine was reported to enhance the barrier functions of TJs in the intestinal cells (Dugan and Mcburney, 1995; Neu et al., 2002; Li et al., 2003). It maintains cldn-1 and occludin protein expression in Caco-2 cell line (Li et al., 2004). GS is regulated by GCs in a tissue specific manner at both transcriptional and post-transcriptional levels (Labow et al., 2001; Chandrasekhar et al., 1999; Sarantos et al., 1994). GCs regulate GS transcription in a rat lung epithelial cell line (Abcouwer et al., 1996), and induce GS in embryonic chicken retina at day 10 (Ben-Dror, 1993; Vardimon et al., 1993; Gorovits et al., 1994). In order to understand the effects of GCs (and other hormones, transcription factors, etc.) on claudin expression, promoter regions of claudins should be cloned and transcription factor binding sites should be identified to determine whether they include any GRE for glucocorticoid activity. However, even though murine cldn 5 includes GRE in its promoter, the regulation of cldn 5 by GCs is cell specific, possibly due to differential activity and distribution of glucocorticoid receptors (Burek and Forster, 2008). In addition to genomic regulation, GCs may modulate the organization of claudins at post-transcriptional level. Therefore, the changes in the distribution of claudins at tight junctional areas in response to GCs at protein level should also be examined at the protein level.

Immunohistochemisrty and quantitative real-time PCR studies on the expression pattern of claudins during embryonic development (presented in Chapter 2) indicate that the formation and stability of TJs in 18-day embryonic intestine is not complete and that TJ are still maturing. Therefore, the effects of DEX on claudin expression would be more pronounced in embryonic than mature tissues according to the previous reports (Peixoto and Collares-Buzato, 2006). Microarray analysis is a very useful technique that can be used to screen the expression of hundreds of genes in a very short time for a given experimental condition. However, it can be easily affected by the technical variations such as dye effect, pin effect and plate effect, etc., and these can hinder the detection of real biological variations. Therefore, the accuracy and detection of fold change and the statistical significance gene expression using microarrays is not as powerful as some of other methods such as quantitative real-time PCR, although each gene was printed 12 times and dye flipping was used to reduce variations from Cy3 and Cy5 dyes in these microarray analyses. Therefore, after screening the gene expression using microarrays, comparative quantitative real-time PCR (QPCR) analyses were used to further analyze the gene expression for cldn-3 and -5. Moreover, in order to increase the sensitivity of detection, the epithelial layer of the embryonic intestine was isolated after organ culture and investigated the effects of different concentrations of DEX at different exposure times. Initially, it had not been detected any changes in cldn-3 and -5 expressions in response to 1μm DEX exposure for 12 hours according to the microarrays data. However, when QPCR was used, dose-dependent increase in both cldn-3 and -5 expressions were detected in response to 12-hour DEX

exposure in the epithelia of embryonic chick intestine. DEX exposure for 24 hours also seems to induce both cldn3 and cldn5 expressions in a dose dependent manner although none of the doses used caused a significant change. This might stem from variation between culture conditions due to possible decline in the stability of cultured tissues or DEX over prolonged periods. Secondly, desensitization of glucocorticoid receptors or a decline in promoter activity of these genes are possible explanations.

To summarize, the characterization of a general GC action on claudin expression and regulation is very complex. GCs exert their effect in a cell specific manner because there are multiple GC receptors for their binding in different cell types. Secondly, the responsiveness of target genes might be different in different tissues and different developmental stages of an organism. Thirdly, the activity of GCs might be modulated by their interactions with other hormones in a tissue specific manner. The diverse effects of GCs have been reported between different species, and even between individuals in the same species. In addition, the expression and organization of claudins are tissue specific and a role of a specific claudin depends on the presence of other claudins or compatibility of claudins to each other.

GENERAL FUTURE DIRECTIONS

The sequences of cldn-1, -3, -5, and -11 are completely known, and another eight claudins have been partially cloned in chicken. The expression patterns of cldn-1 and -3 in young chick embryos have been reported (Simard et al., 2005; Haworth et al., 2005). The presence of some other claudins during embryogenesis is highly possible. Indeed, the

expression profiles of cldn-1, -2, -3, -5 and -16 proteins in late embryonic and hatched chick intestines were determined (see Chapter 2). It is not surprising that some of the claudin family members may have overlapping functions due to high similarities in their aminoacid sequences and topologies. Before understanding the roles and contributions of these important proteins to chick embryonic development, determining the expression patterns of all available claudins during embryogenesis should be done by in situ hybridization, immunohistochemistry, and/or real-time PCR. After that, using over-expression (gain-of-function) and knockout/knockdown (loss of function) studies should be used to indicate the functions of claudins during development of the intestinal epithelium.

Gain-of-function studies can be achieved by overexpression of certain claudins. Recently, cldn-1 overexpression and in-ovo culturing techniques have been successfully applied in chick embryos, and the involvement of cldn-1 in LR patterning has been reported (Simard et al., 2006). Retroviral overexpression of cldn-1 caused an abnormal (leftward) heart looping without affecting the endogenous expression of main asymmetry related genes like Pitx2, Nodal, Shh, and Lefty-1 when this claudin was injected on the right side of the chick embryo at HH stage 4. Likewise, overexpression of Xcla induced the randomization of heart, gut and/or gall bladder during *Xenopus* embryogenesis (Brizuela et al., 2001). Even though these studies might provide us significant information about the involvement of claudins on the LR pattering of internal organs, they do not give us a conclusive result about the function of certain claudins. Overexpression studies should be complemented by loss-of-function studies. Exposed chicks should be further analyzed by whole mount in situ hybridization.

There are multiple ways to perform the loss-of-function of a claudin. Knockout can be performed by homologous recombination mechanism. Cldn-1 knockout mice have been generated, revealing that this protein is crucial for epidermal barrier function of TJs (Furuse et al., 2002). In addition the gene of interest can be knocked-down by morpholino injections (Summerton and Weller, 1997). In this technique, a modified sugar (5-6 membered morpholine ring) binds to 5' leader sequence of mRNA to prevent ribosomes from binding to mRNA; accordingly, this prevents cells from producing the targeted protein. The use of transdominant negative proteins is another strategy to knockdown the action of a specific gene at the protein level by expressing specific inhibitors such as antibodies recognizing the protein of interest. RNAi is a recently discovered technique that can be used in different organisms from plants to mammals to knock-down the expression of a specific gene by delivering double stranded RNA (dsRNA) that corresponds to a segment of the gene of interest (Kjemtrup et al., 1998; Elbashir et al., 2001). RNAi is also considered a defense mechanism of organisms with especially underdeveloped immune systems. RNAi technique has been previously utilized to study the functions of claudins in MDCK cells (Hou et al., 2006). Loss-of- function experiments by RNAi technique is a powerful tool to investigate the functions of claudins and would provide valuable information about the function of these proteins during morphogenesis of various organs such as ovary, digestive and central nervous system organs. Similarly, this application can be use to uncover the roles of other claudins during embryogenesis of the chick. RNA interference (RNAi) can be used in various organisms to knock-down the expression of a specific gene. Unlike the gene knockout (KO) technique, RNAi can be performed easily in shorter time periods and gives

fewer problems with respect to embryonic lethality (Clayton, 2004; Furuse et al., 2002). To study a gene function during development can be challenging without doing in-ovo experiments. Chick embryos provide a convenient model because any desired developmental stage is easily accessible, and the chick embryo can be easily manipulated for RNAi knock-down experiments. There are several methods for siRNA delivery: lipofection, virus-mediated expression, transfection, electroporation, transfection of expression vector including siRNA, and synthetic siRNA injections. Previous studies reported the efficiency of the pSilencer siRNA expression vector having human H1 or U6 RNA polymerase III promoter in the chick embryos (Dai et al., 2005). The placement of the expression vector in the embryos can be monitored by using a modified vector: pEGFP-shRNA whose efficiency has been recently shown in chicken embryos by Dai et al, 2005. This plasmid can co-express enhanced green fluorescent protein (EGFP) and shRNA at the same time, and allowing visualization of the transfection sites. Verification of RNAi with the decrease of both claudin mRNA and protein levels should be done.

REFERENCES

Abcouwer SF, Bode BP, Souba WW. 1995. Glucocorticoids regulate rat glutamine synthetase expression in a tissue-specific manner. J. Surg. Res. 59: 59–65.

Abcouwer SF, Lukaszewicz GC, Souba WW. 1996. Glucocorticoids regulate glutamine synthetase expression in lung epithelial cells. Am. J. Physiol. 270: L141–L151.

Anderson JM, Balda MS, Fanning AS. 1993. The structure and regulation of tight junctions. Curr Opin Cell Biol. 5(5):772-778.

Antonetti DA, Wolpert EB, DeMaio L, Harhaj NS, Scaduto RC. 2002. Hydrocortisone decreases retinal endothelial cell water and solute flux coincident with increased content and decreased phosphorylation of occludin. Journal of Neurochemistry 80:667-677.

Ben-Dror I, Havazelet N, Vardimon L. 1993. Developmental control of glucocorticoid receptor transcriptional activity in embryonic retina. Proc Natl Acad Sci U S A. 90(3):1117-1121.

Black BL, Moog F. 1977. Goblet cells in embryonic intestine accelerated differentiation in culture. Science 197:368-370.

Black BL, Moog F. 1978. Alkaline-phosphatase and maltase activity in embryonic chick intestine in culture - influence of thyroxine and hydrocortisone. Developmental Biology 66:232-249.

Black BL, Smith JE. 1989. Regulation of goblet cell differentiation by calcium in embryonic chick intestine. FASEB J.;3(14):2653-2659.

Black BL. 1978. Morphological development of the epithelium of the embryonic chick intestine in culture: influence of thyroxine and hydrocortisone. Am J Anat.;153(4):573-599.

Black BL. 1988a. Development of glucose active transport in embryonic chick intestine. Influence of thyroxine and hydrocortisone. Comp Biochem Physiol A Comp Physiol. 90(3):379-86.

Black BL. 1988b. Influence of hormones on glycogen and glucose metabolism in embryonic chick intestine. Am J Physiol. 254(1 Pt 1):G65-73.

Boivin MA, Ye D, Kennedy JC, Al-Sadi R, Shepela C, Ma TY. 2007. Mechanism of glucocorticoid regulation of the intestinal tight junction barrier. Am J Physiol Gastrointest Liver Physiol. 292(2):G590-598.

Bossis I, Nishimura S, Muchow M, Porter TE. 2004. Pituitary expression of type I and type II glucocorticoid receptors during chicken embryonic development and their involvement in growth hormone cell differentiation. Endocrinology 145:3523-3531.

Brizuela BJ, Wessely O, DeRobertis EM. 2001. Overexpression of the Xenopus tight-junction protein claudin causes randomization of the left-right body axis. Dev Biol., 230(2):217-229.

Buckingham JC. 2006. Glucocorticoids: exemplars of multi-tasking. British Journal of Pharmacology 147:S258-S268.

Burek M, Förster CY. 2008. Cloning and characterization of the murine claudin-5 promoter. Mol Cell Endocrinol. 298(1-2):19-24.

Buse P, Woo PL, Alexander DB, Reza A, Firestone GL. 1995. Glucocorticoid-induced functional polarity of growth factor responsiveness regulates tight junction dynamics in transformed mammary epithelial tumor cells. J Biol Chem. 270(47):28223-28227.

Chandrasekhar S, Souba WW, Abcouwer SF. 1999. Identification of glucocorticoid-responsive elements that control transcription of rat glutamine synthetase. Am. J. Physiol. 276: L319–L331.

Clayton J. 2004. RNA interference: the silent treatment. Nature. 431(7008):599-605.

Colegio OR, Van Itallie CM, McCrea HJ, Rahner C, Anderson JM. 2002. Claudins create charge-selective channels in the paracellular pathway between epithelial cells. Am J Physiol Cell Physiol. 283(1):C142-147.

Crosnier C, Stamataki D, Lewis J. 2006. Organizing cell renewal in the intestine: stem cells, signals and combinatorial control. Nat Rev Genet. 7(5):349-59.

De Groef B, Grommen SVH, Darras VM. 2008. The chicken embryo as a model for developmental endocrinology: Development of the thyrotropic, corticotropic, and somatotropic axes. Molecular and Cellular Endocrinology 293:17-24.

DeMarco V, Dyess K, Strauss D, West CM, Neu J. Inhibition of glutamine synthetase decreases proliferation of cultured rat intestinal epithelial cells. Nutr. 129(1):57-62.

Dugan ME, McBurney M I. 1995. Luminal glutamine perfusion alters endotoxin-related changes in ileal permeability of the piglet. J. Parenter. Enteral Nutr. 19: 83–87.

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature. 411(6836):494-498.

Felinski EA, Antonetti DA. 2005. Glucocorticoid regulation of endothelial cell tight junction gene expression: novel treatments for diabetic retinopathy. Curr Eye Res. 30(11):949-957.

Fisher DA, 1992. Endocrinology of fetal development. in William's Textbook of Endocrinology. J. D. Wilson and D. W. Foster, eds. W. B. Saunders, Philadelphia, PA. 1049–1077.

Förster C, Burek M, Romero IA, Weksler B, Couraud PO, Drenckhahn D. 2008. Differential effects of hydrocortisone and TNFalpha on tight junction proteins in an in vitro model of the human blood-brain barrier. J Physiol. 586(7):1937-1949.

Forster C, Silwedel C, Golenhofen N, Burek M, Kietz S, Mankertz J, Drenckhahn D. 2005. Occludin as direct target for glucocorticoid-induced improvement of blood-brain barrier properties in a murine in vitro system. Journal of Physiology-London 565:475-486.

Forster C, Waschke J, Burek M, Leers J, Drenckhahn D. 2006. Glucocorticoid effects on mouse microvascular endothelial barrier permeability are brain specific. Journal of Physiology-London 573:413-425.

Furuse M, Hata M, Furuse K, Yoshida Y, Haratake A, Sugitani Y, Noda T, Kubo A, Tsukita S. (2002). Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. J Cell Biol. 156(6):1099-1111.
Gastrointest Liver Physiol. 292(2):G590-598.

Gross KL, Cidlowski JA. Tissue-specific glucocorticoid action: a family affair. Trends Endocrinol Metab. 19(9):331-339.

Guan Y, Woo PL, Rubenstein NM, Firestone GL. 2002. Transforming growth factor-alpha abrogates the glucocorticoid stimulation of tight junction formation and reverses the steroid-induced down-regulation of fascin in rat mammary epithelial tumor cells by a Ras-dependent pathway. Exp Cell Res. 273(1):1-11.

Guyton AC, Hall JE. 1996. The body fluid compartments: extracellular and intracellular fluids; interstitial fluid and edema. In: Guyton AC, Hall JE, editors. Textbook of medical physiology. Philadelphia: WB Saunders Company Press. p. 297-313.

Haworth KE, El-Hanfy A, Prayag S, Healy C, Dietrich S, Sharpe P. 2005. Expression of Claudin-3 during chick development. Gene Expr Patterns. 6(1):40-44.

Hoheisel D, Nitz T, Franke H, Wegener J, Hakvoort A, Tilling T, Galla HJ. 1998. Hydrocortisone reinforces the blood-brain barrier properties in a serum free cell culture system. Biochemical and Biophysical Research Communications 244:312-316.

Hollander D. 1998. Crohn’s disease e a permeability disorder of the tight junction? Gut 29:1621-1624.

Hou J, Gomes AS, Paul DL, Goodenough DA. (2006). Study of claudin function by RNA interference. J Biol Chem. 281(47):36117-23.

Irvine EJ, Marshall JK. 2000. Increased intestinal permeability precedes the onset of Crohn's disease in a subject with familial risk. Gastroenterology. 119: 1740-4.

Irving PM, Gearry RB, Sparrow MP, Gibson PR. 2007. Review article: appropriate use of corticosteroids in Crohn's disease. Alimentary Pharmacology & Therapeutics 26:313-329.

Jenkins SA, Porter TE. 2004. Ontogeny of the hypothalamo-pituitary-adrenocortical axis in the chicken, embryo: a review. Domestic Animal Endocrinology 26:267-275.

Jepson MA, Collares-Buzato CB, Clark MA, Hirst BH, Simmons NL. 1995. Rapid disruption of epithelial barrier function by Salmonella typhimurium is associated with structural modification of intercellular junctions. Infect Immun. 63:356-359.

Kalliecharan R, Hall BK. 1974. Developmental-study of levels of progesterone, corticosterone, cortisol, and cortisone circulating in plasma of chick-embryos. General and Comparative Endocrinology 24:364-372.

Kjemtrup S, Sampson KS, Peele CG, Nguyen LV, Conkling MA, Thompson WF, Robertson D. (1998). Gene silencing from plant DNA carried by a Geminivirus. Plant J.;14(1):91-100.

Labow BI, Souba WW, Abcouwer SF. 2001. Mechanisms governing the expression of the enzymes of glutamine metabolism--glutaminase and glutamine synthetase. J Nutr. 131(9 Suppl):2467S-2474S; discussion 2486S-7S.

Laukoetter MG, Bruewer M, Nusrat A. 2006. Regulation of the intestinal epithelial barrier by the apical junctional complex. Current Opinion in Gastroenterology 22:85-89.
Lett. 344(2):112-116.

Li N, Lewis P, Samuelson D, Liboni K, Neu J. 2004. Glutamine regulates Caco-2 cell tight junction proteins. Am J Physiol Gastrointest Liver Physiol. 287(3):G726-733.

Ma and Anderson, 2006. Tight junctions and intestinal barrier. 2006. In: Johnson LR, editor. Physiology of the gastrointestinal tract. Volume 2, New York: Raven Press. p. 1559-1594.

Madara JL. 1988. Tight junction dynamics: is paracellular transport regulated? Cell. 53(4):497-8.

Marie C. 1981. Ontogenesis of the adrenal glucocorticoids and of the target function of the enzymatic tyrosine-transaminase activity in the chick-embryo. Journal of Endocrinology 90:193-200.

Moog F, Ford E. 1957. Influence of exogenous acth on body weight, adrenal growth, duodenal phosphatase, and liver glycogen in the chick embryo. Anatomical Record 128:592-592.

Moog F, Richardson D. 1955. The functional differentiation of the small intestine .4. the influence of adrenocortical hormones on differentiation and phosphatase synthesis in the duodenum of the chick embryo. Journal of Experimental Zoology 130:29-55.

Nakamura T, Tanabe Y, Hirano H. 1978. Evidence of the in vitro formation of cortisol by the adrenal gland of embryonic and young chickens (Gallus domesticus). Gen Comp Endocrinol. 35:302–308.

Neu, J., DeMarco, V. G. & Li, N. 2002. Glutamine: clinical applications and mechanisms of action. Curr. Opin. Clin. Nutr. Metab. Care 5: 69–75.

Pedernera EA. 1972. Adrenocorticotropic activity in vitro of the chick embryo pituitary gland. Gen Comp Endocrinol 19:589–591

Peixoto EB, Collares-Buzato CB. 2006. Modulation of the epithelial barrier by dexamethasone and prolactin in cultured Madin-Darby canine kidney (MDCK) cells. Cell Biol Int.;30(2):101-113.

Rahner C, Mitic LL, Anderson JM. 2001. Heterogeneity in expression and subcellular localization of claudins 2, 3, 4, and 5 in the rat liver, pancreas, and gut. Gastroenterology. 120(2):411-422.

Romero IA, Couraud PO. 2005. Blood-brain barrier-specific properties of a human adult brain endothelial cell line. Faseb Journal 19:1872-+.

Romero IA, Radewicz K, Jubin E, Michel CC, Greenwood J, Couraud PO, Adamson P. 2003. Changes in cytoskeletal and tight junctional proteins correlate with decreased permeability induced by dexamethasone in cultured rat brain endothelial cells. Neurosci Lett. 344(2):112-116.

Rubbin LL, Staddon JM. 1999. The cell biology of the bloodebrain barrier. Annu Rev Neurosci. 22:11-28.

Rutgeerts P. 2001a. The use of budesonide in the treatment of active Crohn's disease is good clinical practice. Inflamm Bowel Dis. 7(1):60-61.
Rutgeerts PJ. 2001b. Conventional treatment of Crohn’s disease: objectives and outcomes. Inflamm Bowel Dis 7, Suppl 1: S2–S8, 2001.

Sarantos P, Chakrabarti R, Copeland EM, Souba WW. 1994. Dexamethasone increases jejunal glutamine synthetase expression via translational regulation. Am J Surg. 167(1):8-13.

Scholmerich J. 2004. Review article: systemic and topical steroids in inflammatory bowel disease. Aliment Pharmacol Ther 20, Suppl 4: 66–74.

Scott TR, Johnson WA, Satterlee DG, Gildersleeve RP. 1981. Circulating levels of corticosterone in the serum of developing chick-embryos and newly hatched chicks. Poultry science 60:1314-1320.

Simard A, Di Pietro E, Ryan AK. 2005. Gene expression pattern of Claudin-1 during chick embryogenesis. Gene Expr Patterns. 5(4):553-560.

Simard A, Di Pietro E, Young CR, Plaza S, Ryan AK. 2006. Alterations in heart looping induced by overexpression of the tight junction protein Claudin-1 are dependent on its C-terminal cytoplasmic tail. Mech Dev., 123(3):210-227.

Singer KL, Stevenson BR, Woo PL, Firestone GL. 1994. Relationship of serine/threonine phosphorylation/dephosphorylation signaling to glucocorticoid regulation of tight junction permeability and ZO-1 distribution in nontransformed mammary epithelial cells. J Biol Chem. 269(23):16108-16115.

Sonoda N, Furuse M, Sasaki H, Yonemura S, Katahira J, Horiguchi Y, Tsukita S. 1999. Clostridium perfringens enterotoxin fragment removes specific claudins from tight junction strands: Evidence for direct involvement of claudins in tight junction barrier. J Cell Biol. 147(1):195-204.

Stelwagen K, McFadden HA, Demmer J. 1999. Prolactin, alone or in combination with glucocorticoids, enhances tight junction formation and expression of the tight junction protein occludin in mammary cells. Mol Cell Endocrinol. 156(1-2):55-61.

Summerton J, Weller D. 1997. Morpholino Antisense Oligomers: Design, Preparation and Properties. Antisense & Nucleic Acid Drug Development 7. 187-195.

Tedelind S, Ericson LE, Karlsson JO, Nilsson M. 2003. Interferon-gamma down regulates claudin-1 and impairs the epithelial barrier function in primary cultured human thyrocytes. Eur J Endocrinol. 149(3):215-221.

Uni Z, Tako E, Gal-Garber O, Sklan D. 2003. Morphological, molecular, and functional changes in the chicken small intestine of the late-term embryo. Poult Sci. 82(11):1747-1754.

Van Itallie CM, Anderson JM. 2006. Claudins and epithelial paracellular transport. Annu Rev Physiol.; 68:403-429.

Vardimon L, Ben-Dror I, Havazelet N, Fox LE. 1993. Molecular control of glutamine synthetase expression in the developing retina tissue. Dev Dyn. 196(4):276-282.

Wada H. 2008. Glucocorticoids: Mediators of vertebrate ontogenetic transitions. General and Comparative Endocrinology. 156:441-453.

Walsh SV, Hopkins AM, Nusrat A. 2000. Modulation of tight junction structure and function by cytokines. Adv Drug Deliv Rev. 41(3):303-313.

Weiss MD, DeMarco V, Strauss DM, Samuelson DA, Lane ME, Neu J. 1999. Glutamine synthetase: a key enzyme for intestinal epithelial differentiation? JPEN J Parenter Enteral Nutr. 23(3):140-146.

Wild GE, Waschke KA, Bitton A, Thomson AB. 2003. The mechanisms of prednisone inhibition of inflammation in Crohn's disease involve changes in intestinal permeability, mucosal TNFalpha production and nuclear factor kappa B expression. Aliment Pharmacol Ther. 18(3):309-317.

Wise PM, Frye BE. 1973. Functional development of hypothalamo-hypophyseal adrenal cortex axis in chick-embryo, gallus-domesticus. Journal of Experimental Zoology 185:277-291.

Zeissig S, Burgel N, Gunzel D, Richter J, Mankertz J, WahnschaVe U, Kroesen AJ, Zeitz M, Fromm M, Schulzke JD. 2007. Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn’s disease. Gut 56:61–72.

Zettl KS, Sjaastad MD, Riskin PM, Parry G, Machen TE, Firestone GL. 1992. Glucocorticoid-induced formation of tight junctions in mouse mammary epithelial cells in vitro. Proc Natl Acad Sci U S A. 89(19):9069-73.