Tissue expression of claudin-1 and claudin-2 tight junction proteins in chickens[1]

Sydney Stewart, Kelsy Robinson, and Guolong Zhang

Department of Animal Science, Oklahoma State University, Stillwater, OK 74077

ABSTRACT: Understanding gut homeostasis is an active area of research in livestock animals, as it is key to improving animal health and efficiency. Tight junction proteins are responsible for preventing the transport of foreign materials and microorganisms between cells in epithelial and endothelial tissue layers while also regulating the passage of water and ions. Their location and function within these protective "barrier" tissues suggest that tight junction proteins play a significant role in the tissue integrity and innate immunity of the host. The purpose of this study is to examine the expression patterns of mRNAs for different tight junction proteins in various tissues of broiler chickens. Tissue samples were collected from four 30-day old, healthy broiler chickens raised under standard care. Total RNA was isolated, reverse transcribed, and quantitative polymerase chain reaction (qPCR) was performed to measure the mRNA expressions of two major tight junction proteins namely claudin-1 and claudin-2 in the skin, brain, lung, and various segments of the digestive tract. Fold differences among tissue types was calculated using the $\Delta\Delta Ct$ method normalized to the expression of a house-keeping gene, GAPDH. Our results indicated that claudin-1 mRNA was abundant in the skin, lung, spleen, and pancreas, with approximately 250-, 300-, 200-, and 150-fold higher than the brain, respectively. Meanwhile, claudin-2 mRNA was highly expressed in the duodenum, spleen, and pancreas, showing approximately 900-, 650-, and 700-fold higher than the brain, respectively. Understanding the tissue expression patterns of major tight junction proteins represent an important first step in identifying the strategies to modulate their expression in the intestinal tract, thereby improving gut health, immunity, and production efficiency of chickens.

Key words: Claudin, tight junction, poultry, barrier function, innate immunity

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INTRODUCTION

Tight junction proteins are responsible for regulating the transportation of materials between cells in the mucosal layers of epithelial and endothelial tissues (Zuhl et al. 2014). Several families of tight junction proteins have been identified within poultry species, the largest and most well-documented of which is the transmembrane claudin family. The claudins, working in conjunction with other surface-level and transmembrane proteins, control the specificity of tight junction permeability and the ability of small ions to pass through paracellular spaces (Findley et al. 2009). As we continue to study and define the roles of tight junction proteins in epithelial tissues, we find that they play a critical role in maintaining not only homeostasis, but also the health and well-being of the host (Castoldi et al. 2015). Though we have long known that claudins, among other families of tight junction proteins, are present in the epithelial tissues of avian species (Günzel et al. 2013), little to no information is available on where the individual members of these families are most highly expressed. This study focuses on the expression of claudins-1 and -2 in a variety of poultry soft tissues, collected from 30-day old male broiler chickens. In mice, humans, and pigs, claudin-1 is shown to have protective seal-like properties to prevent fluid leakage and bacterial invasion (Tsukita et. al 1998); meanwhile, in mice, dogs and cattle, claudin-2 is shown to form pore-like channels to allow a relatively free exchange of water and ions in the digestive tract and kidneys (Angelow et. al 2009). We believe that knowing where and how particular tight junction proteins are produced in the body of broiler chickens will lead to a better understanding of how these proteins work in conjunction with host immune system. With this knowledge, we hope to take advantage of these proteins' protective function in order to improve poultry health and biosecurity in large-scale production settings without negatively affecting production performance.

MATERIALS AND METHODS

The animal protocols used in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at Oklahoma State University.

Materials

Forward and reverse primers for chicken claudin-1, claudin-2, and GAPDH were purchased from Integrated DNA Technologies, Inc. Plastic 96-well, high-profile PCR plates were manufactured by Bio-Rad Laboratories, Inc. All other reagents, materials, and equipment (unless otherwise noted) were obtained from Thermo-Fisher Scientific.

Animal Care and Tissue Harvest Procedures

Thirty day of hatch male broiler chicks were delivered to the Oklahoma State University Poultry Research Center in Stillwater, Oklahoma from Cobb-Vantress (Siloam Springs, AR). The chicks were sorted into six groups of four and housed under standard care for thirty days, receiving *ad libitum* access to feed and water throughout the study. At Day 30, one of the six groups was selected for harvest and humanely euthanized. Tissues from the proventriculus, duodenum, jejunum, ileum, cecum, colon, pancreas, gallbladder, lung, skin, brain, spleen, and thymus were collected and immediately frozen in liquid nitrogen. The samples were then stored at -80°C until analysis.

RNA Isolation and Real Time Polymerase Chain Reaction (RT-PCR)

The total RNA from tissues was extracted using 750 µL of RNAzol-RT Reagent (Molecular Research Center, Inc.) per sample, per the manufacturer's instructions. Yield and purity of the RNA extracts were measured using a Nanodrop 1000 spectrophotometer. RNA was then diluted to a concentration of 300 ng/µL, and converted to complementary DNA (cDNA) with a Maxima First Strand cDNA Synthesis Kit for RT-qPCR. The RT-PCR reaction was performed in a MJ Research PTC 200 PCR Thermal Cycler (GMI, Inc.) at 25°C for 10 min, 50°C for 20 min, and 85°C for 5 min.

Quantitative Polymerase Chain Reaction (qPCR)

qPCR was performed using Maxima SYBR Green/ROX qPCR Master Mix with the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Inc). Samples were run in duplicate at 1 cycle of 95°C for 10 min; 40 cycles of 94°C for 15 s, 55°C for 20 s, and 72°C for 30 s; and 80 cycles of 55°C for 6 s doe melt curve analysis. Relative gene expression of Claudin-1 and Claudin-2 was calculated and normalized to GAPDH using the ΔΔCt method. Fold change was calculated relative to the tissue with the lowest average Claudin-1 and -2 expression.

RESULTS AND DISCUSSION

Claudin-1 was most highly expressed in skin and lung, followed by spleen and pancreas with fold changes of 264-fold, 287-fold, 172-fold, and 143-fold, respectively (see **Figure 1**). Claudin-1 expression in gallbladder reached approximately 50-fold while all other tissues tested has less than 20-fold increase in mRNA levels relative to brain. The high expression of claudin-1 in the skin and lung is consistent with studies performed in other species, particularly mice and rats. Claudin-1 deficiency in mice causes severe defects in the permeability barrier of the epidermis (Furuse et al. 2001), resulting in rapid postnatal death due to severe water loss and

dehydration (Lal-Nag et al. 2009). This suggests that claudin-1 functions as a protective seal that strictly limits paracellular movement of a few select molecules. Both skin and lung tissues have a large surface area exposed to the outside environment, resulting in increased susceptibility to both evaporative water loss and contact with bacteria, viruses, and other foreign bodies. Thus, one would expect to see increased expression of these "tight"-strand proteins to prevent fluid leakage and invasion of the tissue by foreign bodies (Tsukita et al. 1998).

Maximum claudin-2 expression (see **Figure 2**) was seen in the duodenum (877-fold), spleen (655-fold), and pancreas (727-fold), with fold changes calculated relative to brain. These were followed by ileum (151-fold), lung (304-fold), jejunum (287-fold), and cecum (126-fold) displaying moderate expression while all others had low (<50-fold) claudin-2 mRNA levels. Claudin-2 is classified as a "leaky"-type claudin (Angelow et al. 2009), which forms a pore-like channel to allow relatively free movement of water (Rosenthal et al. 2010) and cations between the intracellular and paracellular spaces (Muto et al. 2010). It appears to be most active in the digestive tract, with decreasing levels of expression in each sequential segment of the small and large intestines. The tissues of the intestines must have the ability to exchange nutrients, water, ions, and waste materials with the lumen space, while also maintaining homeostasis between the host and the commensal resident microbes (Castoldi et al. 2015). Claudin-2's channel-like behavior most likely helps facilitate the movement of ions and water in and out of the extracellular spaces, and protects the tissues from being invaded and overwhelmed by the gut's natural flora.

Claudin-2's role in maintaining the integrity of the gut barrier recently came to light in a study showing that bovine colostrum – a source of vital nutrients and maternal antibodies for the newborn calf – causes an upregulation of claudin-2 expression in the ileal tissues of both cattle and mice (Bodammer et al. 2013). Producers have known for years that adequate consumption of colostrum within the first 12 to 24 hours postpartum is critical for the health and development of the calf, especially for the prevention of gastrointestinal infections resulting in calf scours (Stoltenow et al. 2003). Pathogenic bacteria responsible for causing gastrointestinal infections, such *Vibrio cholerae* and *Clostridium perfringens*, release enterotoxins that specifically target tight junction proteins in order to increase the permeability of the epithelial membrane (Findley et al. 2009; Alshbool et al. 2014; Markov et al. 2014), resulting in the diarrhea and inflammation characteristic to calf scours (among other gastrointestinal diseases). Increased claudin-2 expression as the result of consuming bovine colostrum was shown to strengthen the "gut barrier" of neonatal mice and cattle (Bodammer et al. 2013), resulting in fewer incidences of gastrointestinal disease and infection later on.

Interestingly, we found high expression of both claudin-1 and claudin-2 in the spleen and pancreas. At first glance, it may seem counterintuitive for a secretory organ such as the pancreas to have a high level of "tight"-strand claudin-1 expression, or for any given tissue to have high

expression of both "tight"-strand and "leaky"-strand claudins. However, it is important to note that the permeability of a given tight junction is not dependent on the action of any one particular claudin protein, but rather the coordinated efforts of two or more claudins with other local proteins (Furuse et al. 2002). Furthermore, claudin expression can be influenced by both external conditions, such as environmental salinity (as seen in freshwater versus saltwater fish (Bossus et al. 2015)) and by internal factors, including the age or developmental stage of the host (Li et al. 2014), stress, action by the commensal resident microbes (Ulluwishewa et al. 2011), or infection (Markov et al. 2014). Though we did not observe any signs of stress or illness within our sample birds at the time of harvest, we did see variation in expression levels for the same tissue type (particularly spleen) among the individual birds. We cannot say with absolute certainty that the observed levels of claudin-1 and claudin-2 expression in the tissues of these four particular birds were not influenced by external or internal factors beyond our control.

In the near future, we will expand this study to cover other claudins and members of other tight junction families, as well as expression in other tissue types, ultimately creating a comprehensive "map" of where select proteins are most highly expressed within the male broiler chicken. We hope that an improved understanding of where and how strongly these proteins are expressed will allow us to understand how they work in conjunction with one another, with the cells, and with the host immune system, allowing us to take advantage of their varied functions and open doors for new advances in animal and human health.

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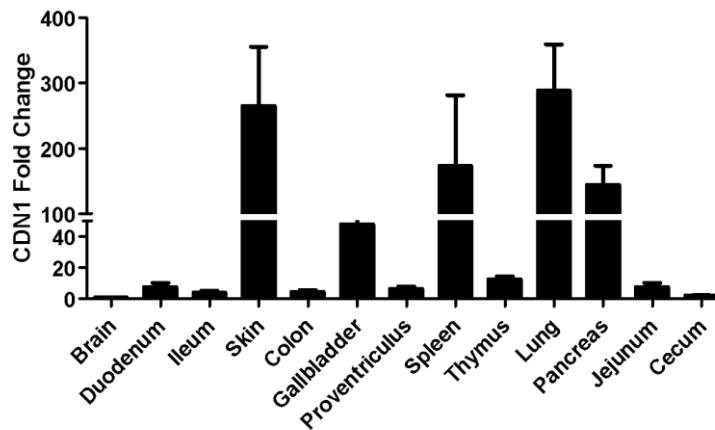
Figures

Fig 1. Claudin-1 expression in chicken tissues. Total RNA was isolated from various tissues in four different 30-day-old healthy chickens, reverse transcribed, and followed by qPCR analysis. Fold differences were calculated using the ΔΔCt method, relative to the brain.
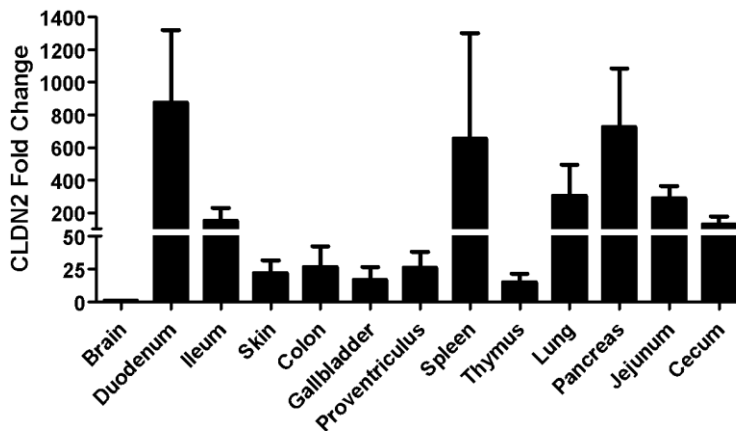

Fig 2. Claudin-2 expression in chicken tissues. Total RNA was isolated from various tissues in four different 30-day-old healthy chickens, reverse transcribed, and followed by qPCR analysis. Fold differences were calculated using the ΔΔCt method, relative to the brain.