

Molecular distribution and localization of extracellular calcium-sensing receptor (CaSR) and vitamin D receptor (VDR) at three different laying stages in laying hens (*Gallus gallus domesticus*)

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ABSTRACT The extracellular calcium-sensing receptor (CaSR) and vitamin D receptor (VDR) play important roles in regulating calcium mobilization, calcium absorption, and calcium homeostasis, and they could be potential therapeutic targets to osteoporosis in laying hens. The present study investigated the molecular distribution of CaSR and VDR and the localization of CaSR in the kidney, proventriculus (true stomach), duodenum, jejunum, ileum, colon, cecum, shell gland, and tibia of laying hens at 3 different laying stages (19, 40, and 55 wk). The results showed that the relative mRNA abundance of CaSR in the kidney, ileum, proventriculus, duodenum, and colon was higher ($P < 0.05$) than the other tissues at 40 and 55 wk. The relative mRNA abundance of CaSR in the tibia was higher ($P < 0.05$) at 55 wk than at 40 wk. However, there were no significant differences in the relative protein abundance of CaSR among all tested tissues at peak

production or in each tissue at the 3 different laying stages ($P > 0.05$). The relative mRNA abundance of VDR was higher ($P < 0.05$) in the small intestine (duodenum, jejunum, and ileum) when compared with other tissues at the 3 different laying stages. The relative protein abundance of VDR in the duodenum was higher ($P < 0.05$) than that in the proventriculus, colon, and cecum. There were no significant differences in the VDR expression among the tested tissues at the 3 different laying stages ($P > 0.05$). The immunohistochemical results showed that the positive staining was found widely in each tissue. Moreover, different laying stages did not affect the localization of CaSR except for the tibia tissue. In conclusion, similar to VDR, CaSR was widely expressed not only in the gut but also in the tibia and shell gland in laying hens. The expression level of CaSR and VDR in all tested tissues was unchanged at the different laying stages.

Key words: calcium-sensing receptor, vitamin D receptor, localization, distribution, laying hen

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INTRODUCTION

Laying hens have a dynamic bone turnover associated with the daily egg-laying cycle. Because of rapid bone turnover and calcium mobilization from bones for eggshell formation, osteoporosis is a big challenge in the laying hen industry caused by the progressive loss of structural bone (Whitehead and Fleming, 2000). Osteoporosis is one of the major leading causes of bone

fractures in older laying hens, which are associated with pain, reduced egg production and eggshell quality, and animal welfare issues (Knowles and Wilkins, 1998; Cransberg et al., 2001). Calcium is the most important mineral in maintaining the structural integrity of bones and eggshells (Olgun and Aygun, 2016). Approximately 2.4 g of calcium is required in approximately 20 h for a laying hen to calcify a 60 g shelled egg in the shell gland, which is a principle calcium-consuming organ and is unique in avian species (Fleming, 2008). Only 60% to 75% of the eggshell calcium can be provided by the feed and the remainder must be from the nonstructural medullary bone (Fleming, 2008). Also, calcium absorption, femur ash, and eggshell quality decline with age (Al-Batshan et al., 1994). Therefore, it is critical to maximize skeletal mineralization through calcium supplementation in laying hen diets before sexual maturity,

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so they have sufficient calcium or bone density to support egg laying. However, most dietary interventions are ineffective during the laying period. Calcium deficiency affects eggshell quality and also stimulates the secretion of parathyroid hormone (PTH) and vitamin D synthesis, which in turn leads to the depletion of calcium in the bone (Rath et al., 2000). Hypocalcemia or inadequate calcium levels in the blood are likely to decrease bone strength (Rath et al., 2000). An excess of calcium decreases phosphorous availability and reduces feed intake, which negatively affects eggshell quality and bone strength. Taken together, restoring or maintaining the desired calcium homeostasis by nutritional solutions might be an effective strategy to improve bone health and eggshell quality in laying hens.

Extensive research has been done on the topic of calcium in laying hens. However, most studies have focused on the effects of dietary calcium sources, requirements, particle sizes, and calcium: phosphorus ratio on laying hen performance and health (Keshavarz, 2003; Cufadar et al., 2011; Ganjigohari et al., 2018). Osteoporosis cannot be easily solved by simply increasing dietary calcium contents, and it is one of the common diseases in laying hens globally. Calcium homeostasis is regulated through PTH, active form of vitamin D₃, calcitonin, vitamin D receptor (VDR), calcium transporters, and calcium-sensing receptor (CaSR) (Proszkowiec-Weglarz and Angel, 2013). CaSR is a homodimeric complex located in the cell membrane and belongs to class C G-protein-coupled receptor (GPCR) (Jensen and Brauner-Osborne, 2007). It can sense subtle changes in extracellular calcium concentration and thus mediating PTH secretion to maintain calcium homeostasis via regulating intestinal absorption, bone storage and exchange, and renal reabsorption (Conigrave, 2016). In addition, CaSR mediates a variety of physiological and pathophysiological processes such as ion channel activity, gene expression, inflammation, proliferation, differentiation, and apoptosis via inducing downstream signaling cascades. Chicken CaSR (cCaSR) has 79% and 84% homology with human CaSR on the nucleotide and amino acid level, respectively. The in situ hybridization has revealed that CaSR is present in the parathyroid, kidney, brain, and small intestine (Diaz et al., 1997). Yarden et al. (2000) have showed that the expression of cCaSR in the parathyroid gland is inversely associated with changes in plasma calcium concentration. Chickens fed vitamin-D-deficient diets with a low CaSR expression were characterized by the highest concentration of PTH, whereas high CaSR gene expression level in vitamin-D-depleted chickens was associated with low PTH content in the parathyroid gland (Yarden et al., 2000). All these results indicate that the functional CaSR in chickens possesses similar characteristics to mammalian CaSR and may play very important roles in avian calcium homeostasis.

It has been reported that CaSR is widely expressed in multiple tissues including the parathyroid gland, kidney, bone, and gastrointestinal tract (GIT) in humans, rat,

and swine (Abukawa et al., 2001; Kos et al., 2003; Quarles, 2003; Al-Dujaili et al., 2016; Zhao et al., 2019). CaSR also plays a central role in human calcium homeostasis by influencing bone modeling and remodeling and recently becoming a potential therapeutic target to osteoporosis in postmenopausal women (Halse et al., 2014; Liang et al., 2016). However, it is not clear whether CaSR is expressed in the proventriculus, bone, and shell gland in laying hens. It has been demonstrated that chicken VDR (cVDR) is expressed in the kidney (Lu et al., 1997), intestine (Lu et al., 1997), and shell gland (Yoshimura et al., 1997) of laying hens. We hypothesized that similar to cVDR, cCaSR is expressed in the proventriculus, shell glands, and bones in laying hens. The objective of the present study was to investigate the molecular distribution and localization of poultry CaSR and VDR in the kidney, GIT, shell gland, and bone in laying hens at 3 different laying stages (peak laying, middle laying, and late laying).

MATERIALS AND METHODS

Locus and Structure of Chicken (Gallus gallus) CaSR

The nucleotide sequence and amino acid sequence information of cCaSR was collected from the National Center for Biotechnology Information (NCBI, Genbank Accession No: XM_416491.6 and XP_416491.5) and Ensembl genome database (ENSGALG00000038405). The schematic diagram of the cCaSR gene exon/intron organization and protein domains was drawn using an online tool Exon-Intron Graphic Maker (<http://www.wormweb.org/exonintron>). The secondary structure diagram of the cCaSR was constructed using PROTTER (<http://wlab.ethz.ch/protter/start/>). The predicted 3-dimensional model of a cCaSR disulfide-linked homodimeric extracellular domain (ECD) structure was based on a human CaSR template (Geng et al., 2016) using the SWISS-MODEL (<https://swissmodel.expasy.org/interactive>).

Animals and Management

A total of 18 Lohmann laying hens at 19 wk (6 birds), 40 wk (6 birds), and 55 wk (6 birds) of age were obtained from the Poultry Unit, Glenlea Research Station, University of Manitoba. Birds had ad libitum access to feed and water, and they were housed and maintained in accordance with the Canadian Council on Animal Care (CCAC) guidelines for the care and use of farm animals in research, teaching, and testing (CCAC, 2009). The experimental protocol (F18-043) was approved by the Fort Garry Campus Animal Care Committee at the University of Manitoba. Hens were fed a mash diet that was formulated to meet or exceed the nutritional requirements specified in the management guide (Lohmann Tierzucht).

Tissue Collection and Preparation

Six birds were euthanized using carbon dioxide asphyxiation at 19, 40, and 55 wk of the laying period, respectively. Kidney, proventriculus (true stomach), duodenum, jejunum, ileum, colon, cecum, shell gland, and tibia were collected. Intestinal contents were flushed with ice-cold phosphate-buffered saline (**PBS**). Collected tissues were immediately frozen in liquid nitrogen and then stored at -80°C . Frozen samples were then individually ground to a fine powder using liquid nitrogen and a tissue pulverizer (59013N, Cole-Parmer, Vernon Hills, IL, USA) for RNA and protein extraction. Tissues were fixed in a 10% buffered formalin (Fisher Scientific, Waltham, MA, USA) for 24 h, and then formalin was replaced with 70% ethanol for longer storage for the paraffin block preparation.

Real-Time Quantitative Polymerase Chain Reaction (Real-Time RT-qPCR)

Total RNA extraction from each tissue was conducted using TRIzol reagent (Invitrogen Life Technologies, Waltham, MA, USA) as described in the manufacturer's instructions. The quantity and quality of RNA were analyzed by Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and the integrity of RNA was checked by RNA electrophoresis in agarose gel. Next, the first-strand cDNA was synthesized using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) according to the corresponding manufacturer's protocol. The relative mRNA abundances of CaSR and VDR were measured using SYBR Green Supermix (Bio-Rad) by a CFX Connect Real-Time PCR Detection System (Bio-Rad). PCR amplification was performed at a set of 3 min at 95°C , then 40 cycles of 20 s at 95°C , 30 s at 60°C , and 30 s at 72°C . At the end of each cycle, the fluorescence was monitored for 10 s. Each reaction was completed with a melting curve analysis to ensure the specificity of the reaction. Primers used in this study are listed in [Table 1](#). Relative gene expression was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method ([Livak and Schmittgen, 2001](#)). Because of the big gap of threshold cycle (Ct) value

for the reference gene when comparing tibia with other tissues, the target gene expression of tibia was shown separately.

Western Blot Analysis

The relative protein expression of CaSR and VDR was determined by western blot analysis. Ground tissue samples were homogenized and lysed by a homogenizer (Powergen 125, Fisher Scientific) using precooled RIPA buffer containing Halt protease and phosphatase inhibitor cocktail (Thermo Scientific) on ice. Sample lysates were centrifuged at $12,500 \times g$ for 15 min to remove the insoluble debris. Total protein concentration was determined using the Pierce BCA protein assay kit (Waltham, MA, USA) as described in the manufacturer's instructions. Protein was then denatured using 4x Laemmli sample buffer (Bio-Rad) with 0.1 M dithiothreitol (**DTT**) at 95°C for 9 min, the denatured protein samples (70 μg) were loaded and separated by 4 to 15% Mini-PROTEAN TGX Stain-Free Protein Gels (Bio-Rad) and transferred onto a polyvinylidene difluoride (**PVDF**, Bio-Rad) membrane. Each membrane was blocked using 5% skim milk powder dissolved in tris-buffered saline with 0.1% Tween 20 (**TBST**) for 1 h at room temperature and then incubated with the rabbit polyclonal anti-CaSR antibody (1:1000, ab137408, Abcam, Cambridge, MA, USA) and VDR Monoclonal Antibody (9A7) (1:2000, MA1-710, Thermo Scientific) at 4°C overnight. Secondary antibody incubation used horseradish-peroxidase-conjugated goat anti-rabbit IgG (65-6120, 1:1000, Thermo Scientific) and horseradish-peroxidase-conjugated anti-rat IgG (112-035-003, 1:5000, Jackson Immuno Research Laboratories, West Grove, PA, USA). Images were detected by ChemiDoc Imaging Systems (Bio-Rad) after incubation with Clarity Max Western ECL Substrate (Bio-Rad). The densitometry of the target protein bands was quantified by Image Lab Software (Bio-Rad) and normalized to the total protein. The quantitative results were presented as relative to the kidney (in different tissues) at the 40 wk of age or relative to the 19 wk of age (at different ages) in each tissue.

Table 1. Primers used in this study.

Genes	Genbank Accession No.	Primer sequences (5' → 3')
<i>cCaSR</i>	XM_416491.6	Forward: CAAACCAACGGGCACAGAAG Reverse: ATGCACTCCACTGATTTCGGG
<i>cVDR</i>	AF011356.1	Forward: CCGGATTTCAGGGATCTGACG Reverse: AAGTCATTGCTTCCGAGGT
<i>cGAPDH</i> (Reference)	NM_204305	Forward: ACTGTCAAGGCTGAGAACGG Reverse: CACCTGCATCTGCCCATTTG

Note: cCaSR: chicken calcium-sensing receptor; cVDR: chicken vitamin D receptor; cGAPDH: chicken GAPDH.

Immunohistochemistry

Fixed tissues were processed and embedded in paraffin according to our previously published method (Zhao et al., 2019). Paraffin blocks were sectioned at 5 μm , and 2 sections were placed on each Superfrost plus microscope slide (Fisher Scientific). Slides were then deparaffinized in xylene twice and then rehydrated in ethanol diluted serially in Mili-Q water (100, 90, 80 and 70%) and finally rinsed in Mili-Q water. Firstly, sections were stained with hematoxylin and eosin (H&E) to check the morphological state of the tissues. For immunohistochemical staining, antigen retrieval was conducted by incubating slides in 10 mmol sodium citrate with 0.5% Tween 20 (pH at 6) in a water bath at 95°C for 10 min. Sections were incubated with a mixture of 3% hydrogen peroxide and 10% methane for 10 min to block endogenous peroxidases, followed by the incubation with Avidin/Biotin Blocking system (Biolegend, San Diego, CA, USA) according to the manufacturer's instruction, and then sections were blocked with normal donkey serum (1:20, 017-000-001, Jackson Immuno Research Laboratories) for 1 h. Sections were incubated with a rabbit polyclonal anti-CaSR antibody (1:50, ab137408, Abcam) diluted in 2% bovine serum albumin (BSA) overnight at 4°C. Next, Biotin-SP-AffiniPure Donkey Anti-Rabbit IgG (H + L) (1:500, 711-065-152, Jackson ImmunoResearch Laboratories) was incubated for 1 h at room temperature and followed by the peroxidase-conjugated streptavidin incubation (1:500, Jackson ImmunoResearch Laboratories) at room temperature for 30 min. The color was visualized using Pierce DAB Substrate Kit (PI34002, Thermo scientific) and counterstained with Mayer's Hematoxylin Solution (Sigma-Aldrich, St. Louis, MO, USA). Sections incubated without primary antibody were set as a negative control. Slides were finally scanned and photographed by using a Zeiss Axio Scope (Carl-Zeiss Ltd., Jena, Germany).

Statistical Analysis

Statistical analyses were carried out using SAS 9.4 (the SAS Institute, Cary, NC) and all figures were made using GraphPad Prism 6 software (San Diego, CA, USA). Results were expressed as the mean \pm SEM. Statistical significance was determined

using one-way ANOVA followed by Tukey's multiple-comparison test. P value < 0.05 was considered statistically significant.

RESULTS

Structure and Hydrophobicity Analysis of cCaSR

Based on the information obtained from NCBI and Ensembl genome database, a schematic diagram of the *cCaSR* gene exon/intron and protein domains is shown in Figure 1. The *cCaSR* gene that maps the chicken chromosome 1q has 7 exons, spanning ~ 42 kb. The T-cell antigen *CD86* gene lies upstream and cysteine protease inhibitor *CSTB* gene lies downstream of the *cCaSR* gene. Exons 1–7 encode 1059 amino acids (AAs) of *cCaSR* protein; ECD is encoded by 1,833 nucleotides of exons 1–6 including signal peptides encoded by 57 nucleotides of exon 1; transmembrane domain (TMD) is encoded by 747 nucleotides of exon 6; and intracellular domain (ICD) is encoded by 594 nucleotides of exon 7. The schematic diagram of the secondary structure of *cCaSR* protein is shown in Figure 2. The *cCaSR* consists of 1,059 AAs and divides into 3 domains: 1) a large N-terminal ECD that comprises the first 611 AAs containing a 19 AA N-terminal signal peptide; 2) a 7-helical TMD that comprises 250 AAs; and 3) a C-terminal ICD that comprises 198 AAs. The 3-dimensional model of a *cCaSR* ECD structure was predicted based on a human CaSR template using SWISS-MODEL (Figure 3). The *cCaSR* functions in the form of disulfide-linked homodimers. The ECD is the main site where ligands bind, consisting of a large extracellular Venus Flytrap (VFT) module (Lobe1 and Lobe 2) and a cysteine-rich (CR) domain, which connects the VFT module to the TMD.

Relative mRNA Abundance of cCaSR and cVDR

The relative mRNA abundance of *cCaSR* and *cVDR* in the kidney, proventriculus, duodenum, jejunum, ileum, colon, cecum, shell gland, and tibia was shown in Figure 4. The results showed that the mRNA of *cCaSR* and *cVDR* was widely expressed in the kidney, proventriculus, gut, bone, and shell gland of laying

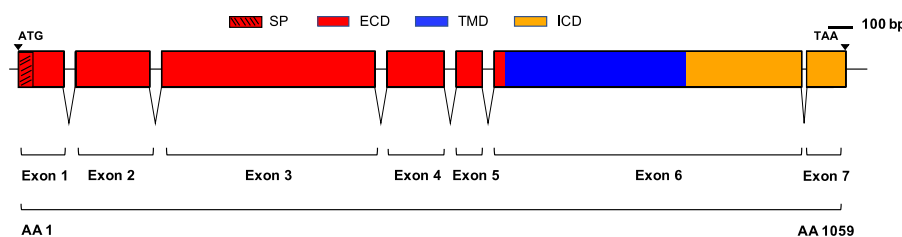


Figure 1. Schematic diagram of the chicken CaSR (*cCaSR*) gene exon/intron organization and protein domains. Exons were drawn to the scale, but introns were not. There are 7 exons and 6 introns in the *cCaSR* gene. Filled bars represent mRNA coding regions including exons 1–7. ATG, initiation codon; TAA, stop codon. The *cCaSR* protein contains 1,059 amino acid residues. Abbreviations: ECD, extracellular domain; ICD, intracellular domain; SP, signal peptide; TMD, transmembrane domain.

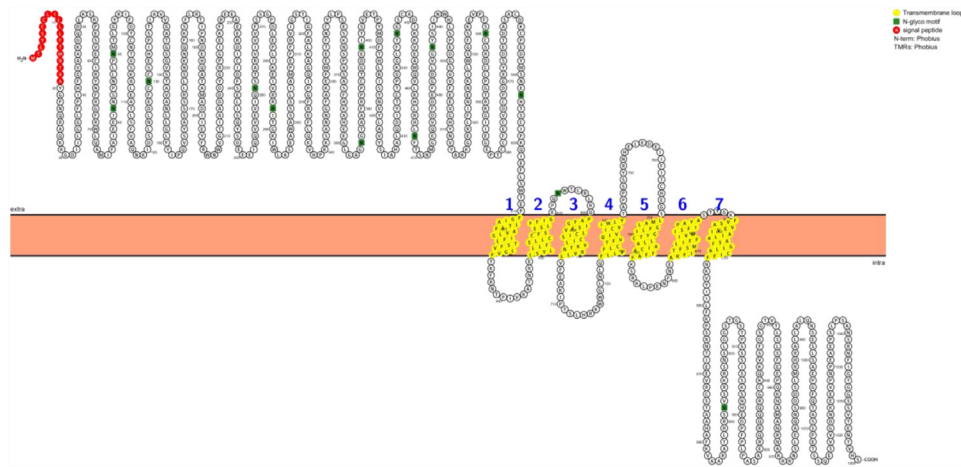


Figure 2. Schematic diagram of the structure of chicken calcium-sensing receptor exported by PROTTTER tool based on the protein sequence obtained from NCBI (Accession No: XP_416491.5).

hens. The relative mRNA abundance of cCaSR in the cecum was significantly higher at 19 than at 40 and 55 wk ($P < 0.05$). The relative mRNA abundance of cCaSR in the kidney, ileum, proventriculus, duodenum, and colon was significantly higher than that in other tissues at 40 wk ($P < 0.05$). In the tibia, the relative mRNA abundance of cCaSR was significantly higher at 55 than that at 40 wk ($P < 0.05$). The relative mRNA abundance of cVDR was at a relatively high level in the small intestine (duodenum, jejunum, and ileum) when compared with other tissues at the 3 different laying stages, but these differences were not significant. Moreover, the relative expression of cVDR in the shell gland was significantly higher at 40 than at 19 and 55 wk ($P < 0.05$).

Relative Protein Expression of cCaSR and cVDR

As shown in Figure 5, the protein of cCaSR and cVDR was widely expressed in the kidney, proventriculus, gut,

bone and shell gland of laying hens at 40 wk. Western blot images show that cCaSR are expressed in 4 bands with varied molecular weights (Figure 5A), and the bands for quantitating are indicated in “*”: 1) more than 250 kDa; 2) between 150 and 250 kDa; 3) and 4) below 150 kDa. The band of chicken VDR was exhibited between 50 and 75 kDa (around 60 kDa) in each tissue (Figure 5A). Figure 5B shows the quantified number of the 4 bands of cCaSR. The relative cCaSR protein abundance was unchanged ($P > 0.05$) in the different organs at 40 wk. Figure 5C shows quantitative relative expression of cVDR. The relative cVDR protein abundance was significantly higher in the duodenum than in the proventriculus, colon, and cecum ($P < 0.05$) at 40 wk. However, no significant difference was observed among the kidney, proventriculus, jejunum, ileum, colon, cecum, and shell gland at 40 wk ($P > 0.05$). As shown in Figure 6A to 6R, the protein of cCaSR and cVDR was expressed at the different ages of laying hens. No significant differences in the relative protein abundance of cCaSR and cVDR were observed among tissues at the 3 different laying stages ($P > 0.05$).

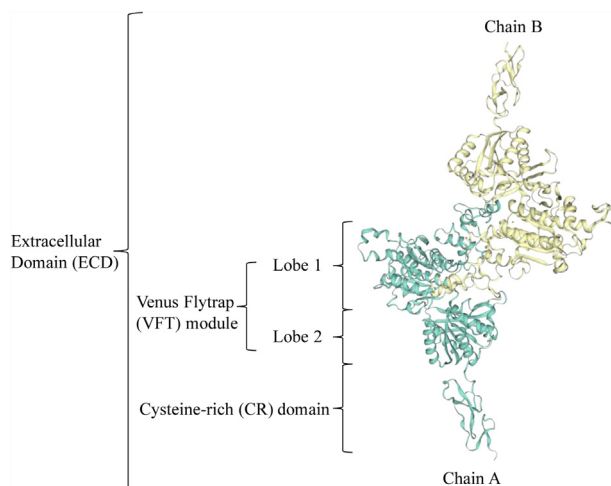


Figure 3. Prediction 3-dimensional model of a chicken calcium-sensing receptor disulfide-linked homodimeric extracellular domain structure based on a human calcium-sensing receptor template using the SWISS-MODEL.

Localization of cCaSR

The localization of cCaSR in the kidney, proventriculus, gut, shell gland, and tibia at different laying stages was detected by immunohistochemical staining. As shown in Figure 7, the positive brown staining was found widely in each tissue at different laying stages. In the kidney, the positive cCaSR staining was widely detected in the nephron including the renal tubules and corpuscles. More specifically, in the renal tubules, the positive stain was only detected in the proximal convoluted tubules instead of distal tubules; in the renal corpuscle, the positive stain was only detected in the glomerulus. In the proventriculus, cCaSR was distributed on the surface of serrated secretory tubules. In the small intestine including duodenum, jejunum, and ileum, the positive staining was distributed widely along the simple columnar epithelium of the villus and crypts. In the large

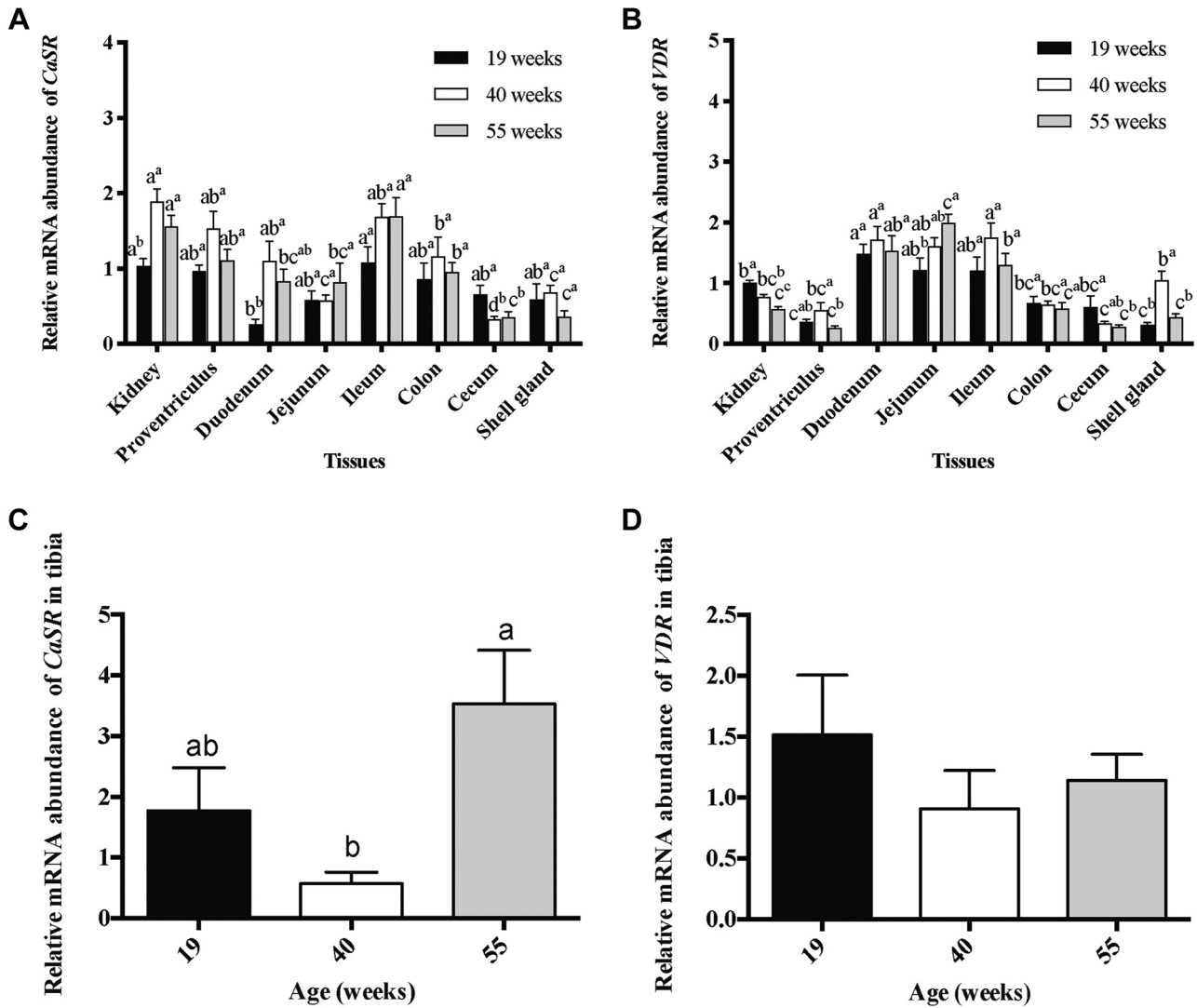


Figure 4. The relative mRNA abundance of chicken calcium-sensing receptor (cCaSR, A) and chicken vitamin D receptor (cVDR, B) in different tissues at 3 laying stages, and the relative mRNA abundance of cCaSR (C) and cVDR (D) in the tibia at 3 different laying stages. Data were presented as mean \pm SEM, $n = 6$. Different letters represent a significant difference among different tissues, and different letter superscripts represent a significant difference at different laying stages ($P < 0.05$).

intestine including colon and cecum, the positive staining was mainly seen in the epithelium of crypts of Lieberkühn. In the shell gland, immunohistochemical positivity was primarily found in the stratified cuboidal epithelium. In the tibia, the clear immunohistochemical positivity was distributed within different laying stages. As circled in red, medullary bone tissues were only observed in 40- and 55-week-old layers, which might be related to sexual maturity. The positive staining was mainly detected in compact structural bone (cortical bone) at 19 wk of age and mainly in the medullary bone at the 40 wk of age. At 55 wk of age, the immunohistochemical positivity was found in both medullary bone and cortical bone.

DISCUSSION

Previous studies suggested that cCaSR has similar characteristics with mammalian CaSR and may also

play very important roles in avian calcium homeostasis because of the close conservation of the amino acid sequence of the cCaSR with human CaSR (Diaz et al., 1997) and other mammalian CaSR (Brown et al., 1993; Riccardi et al., 1995; Zhao et al., 2019). Also, the expression of cCaSR in the parathyroid gland is regulated by the plasma calcium concentrations and vitamin D level in the diet (Yarden et al., 2000). In the present study, cCaSR was predicted to show a similar secondary and 3-dimensional crystal structure with mammalian CaSR (Diaz et al., 1997), and the cCaSR was able to respond to exogenous Ca^{2+} , Mg^{2+} , and Gd^{3+} (Diaz et al., 1997) which are evidence to support the hypothesis that cCaSR could play similar roles as mammalian CaSR. It has been well documented that in addition to calcium, a variety of nutritional ligands including di- and trivalent cations, AAs, pharmacological agents, polyamines, and polypeptides in mammals can activate CaSR (Magno et al., 2011; Zhao et al., 2019). Therefore,

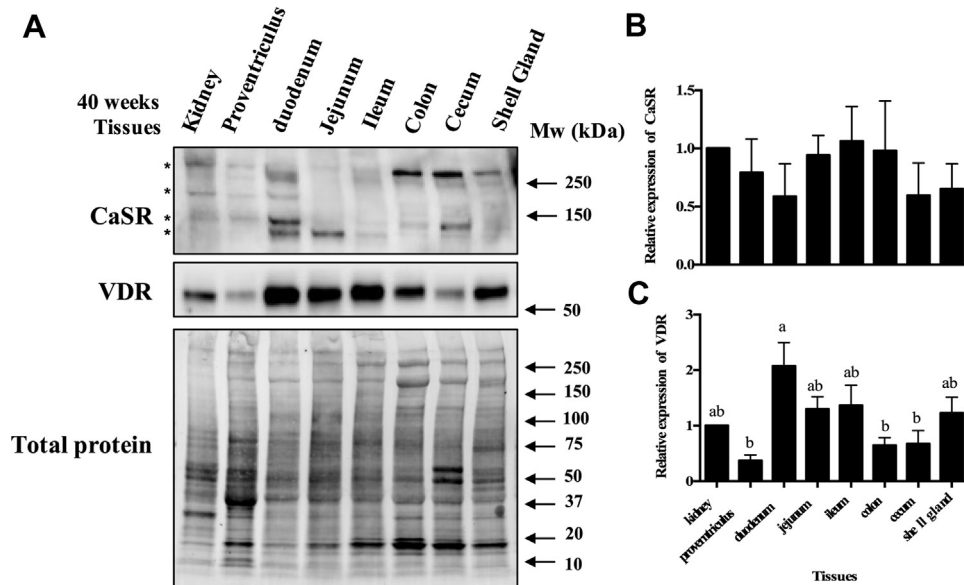


Figure 5. Representative western blot images (A) of chicken calcium-sensing receptor (cCaSR) and chicken vitamin D receptor (cVDR); the quantitative relative expression of cCaSR (B) and cVDR (C) in different tissues at the 40 wk of age (peak production). Data were presented as mean \pm SEM, $n = 6$. Different letters represent a significant difference ($P < 0.05$).

the cCaSR may share similar ligands with the mammal CaSR. The cCaSR has a large hydrophilic ECD, a 7-helical hydrophobic TMD, and a small hydrophilic ICD, and functions to detect ionized plasma calcium concentration, which shows a similar variation range to mammals, of between 1.2 mmol and 1.3 mmol to maintain calcium homeostasis (Diaz et al., 1997). Therefore, it would be critical to characterize the functions of cCaSR and its potential ligands including nutrients, nonnutritional compounds, and tastants, which might help to develop novel approaches to reduce the incidence of osteoporosis in laying hens.

The distribution of cCaSR and cVDR is closely related to their function. In this study, the results demonstrated that cCaSR and cVDR were expressed in the kidney, proventriculus, duodenum, jejunum, ileum, colon, and cecum, which is consistent with the cCaSR expression profile from previous studies in the kidney, duodenum, and the whole small intestine (Diaz et al., 1997; Deng et al., 2010). Notably, the gene and protein expression, and the distribution pattern of cCaSR in the different segments of the GIT, shell gland, and tibia, was reported for the first time in the current study. Moreover, our results demonstrated that similar to cVDR, cCaSR is also distributed in the shell glands and tibia of laying hens, similar to what has been reported by others (Berry et al., 1996; Yoshimura et al., 1997), but they were expressed with different abundance. Similar to mammalian CaSR, the expression of cCaSR in the bones may be related to the calcium homeostasis and bone turnover in laying hens. However, the role of cCaSR in the shell glands is not known but may be related to eggshell formation because the shell gland is involved in the synthesis and secretion of substances for the formation of distinct layers of the eggshell (Bar, 2009).

In the present study, the higher expression of cCaSR was found in the kidney, proventriculus, and small

intestine especially the ileum, which is consistent with that in mammals (Hebert, 1996; Brown and MacLeod, 2001; Fudge and Kovacs, 2004; Hebert et al., 2004; Zhao et al., 2019). However, the higher expression of cVDR was only found in the small intestine (duodenum, jejunum, and ileum), which is consistent with its function. CaSR maintains calcium homeostasis by regulating parathyroid gland, kidney, GIT, and the secretion of PTH, calcitonin, and $1,25(\text{OH})_2\text{D}_3$. The kidney is responsible for the reabsorption of calcium and other mineral ions such as magnesium and to the amount of minerals being excreted. Moreover, renal calcium status is a part of calcium homeostasis that closely affects homeostatic regulation of calcium and the secretion of its regulators (PTH, calcitonin, and $1,25(\text{OH})_2\text{D}_3$) because of feedback mechanisms (Riccardi and Brown, 2010). Therefore, detecting and monitoring renal calcium levels is critical to systematically regulate calcium homeostasis. Lots of evidence showed that CaSR is widely distributed along the epithelium of the GIT where digestion and absorption of nutrients occur (Cheng et al., 1999; Hebert et al., 2004). Changes in Ca^{2+} concentration are also associated with the secretion of gastrin, gastric acid, and HCO_3^- in the stomach, which is important for food digestion (Cheng et al., 1999). The proventriculus is the true stomach in poultry and performs the same function as the stomach in mammals, which can secrete gastric acid and enzymes to initiate protein digestion. Proteins are broken down by proteases into smaller peptides and AAs during the digestion. L-AAs and other small peptides are known to be potent ligands for CaSR. This might be the reason why the expression level of cCaSR was much higher in the proventriculus. The small intestine undertakes the most absorption and chemosensing of nutrients. The gut chemosensing system is considered to be performed by the GPCRs

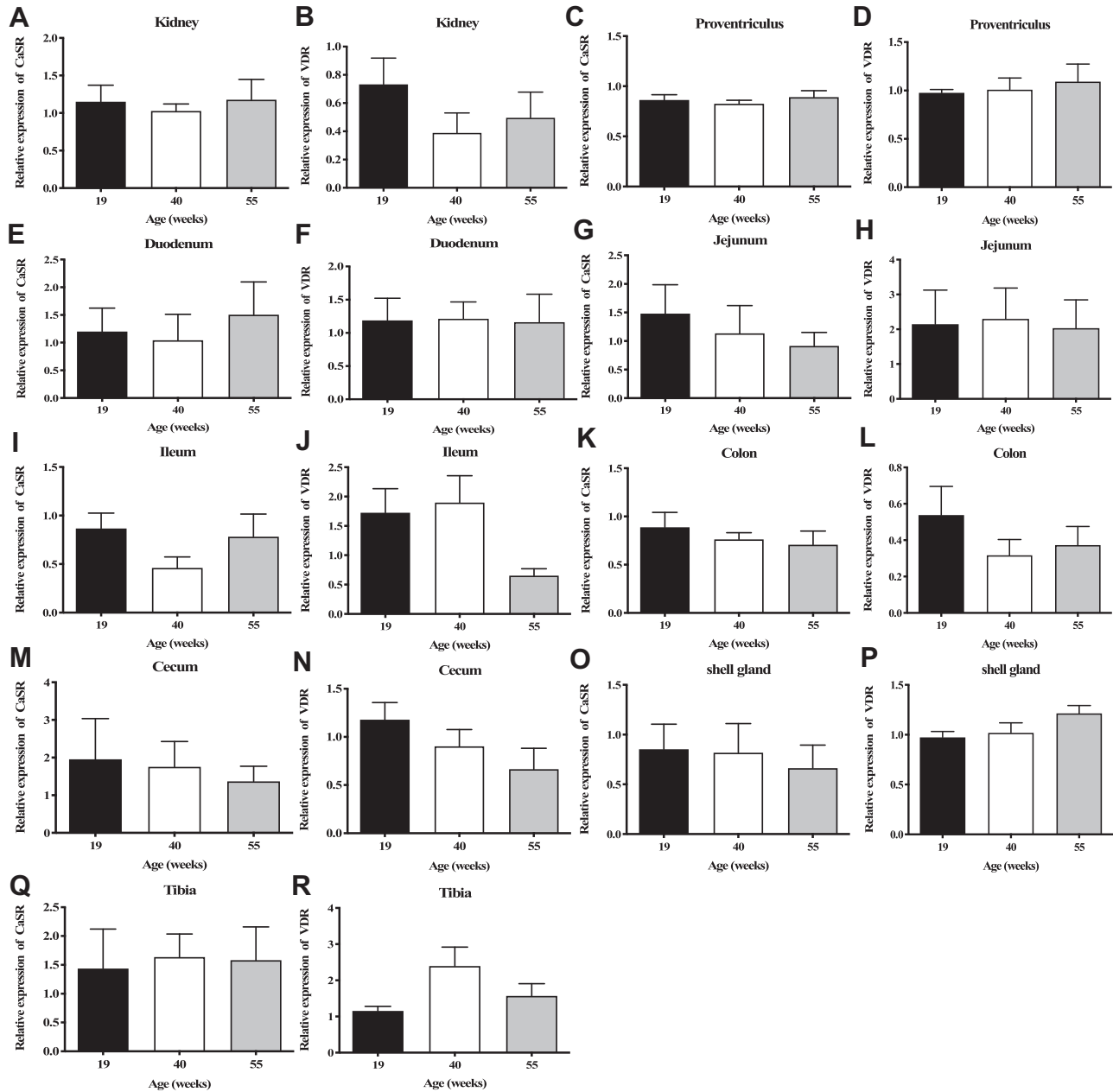


Figure 6. The relative expression of chicken calcium-sensing receptor in the kidney (A), proventriculus (C), duodenum (E), jejunum (G), ileum (I), colon (K), cecum (M), shell gland (O), and tibia (Q) and chicken vitamin D receptor in the kidney (B), proventriculus (D), duodenum (F), jejunum (H), ileum (J), colon (L), cecum (N), shell gland (P), and tibia (R) at 3 different laying stages. Data were presented as mean \pm SEM, $n = 6$.

that recognize nutrients, chemicals, and even microorganisms, and then initiates a downstream signaling cascade to maintain mineral homeostasis and to regulate feed intake, gut function, and metabolism (Roura et al., 2019). Also, CaSR as a multimodal sensor can bind many nutrients especially Ca^{2+} and L-AAAs, which might mediate nutrient absorption. Within the small intestine, unlike in mammals, the majority of calcium absorption does not take place in the ileum in laying hens (Hurwitz and Bar, 1966), but the expression of cCaSR in the ileum was higher than that in the duodenum and jejunum, which was consistent with the results of CaSR distribution in pigs (Zhao et al., 2019). This might be attributed to more hormones secreted by ileal enteroendocrine cells, which depends on the

signaling of cellular calcium mobilization (Liou, 2013). There are various hormones such as glucagon-like peptide 1 and 2, peptide YY, and oxyntomodulin that are secreted by L cells in the ileum, regulating satiety, intestinal motility, transit, gastric emptying, intestinal integrity, and permeability, etc. (Liou, 2013). These results also illustrated that the predominant role of cCaSR might not be to directly regulate calcium absorption. Instead, the principle function of cCaSR is probably sensing the changes of calcium concentration to communicate with other receptors and mediate various biological processes via hormones or downstream signaling pathways to maintain the homeostasis of calcium or other nutrients. Compared with CaSR, the function of VDR is more specific, which is assisting

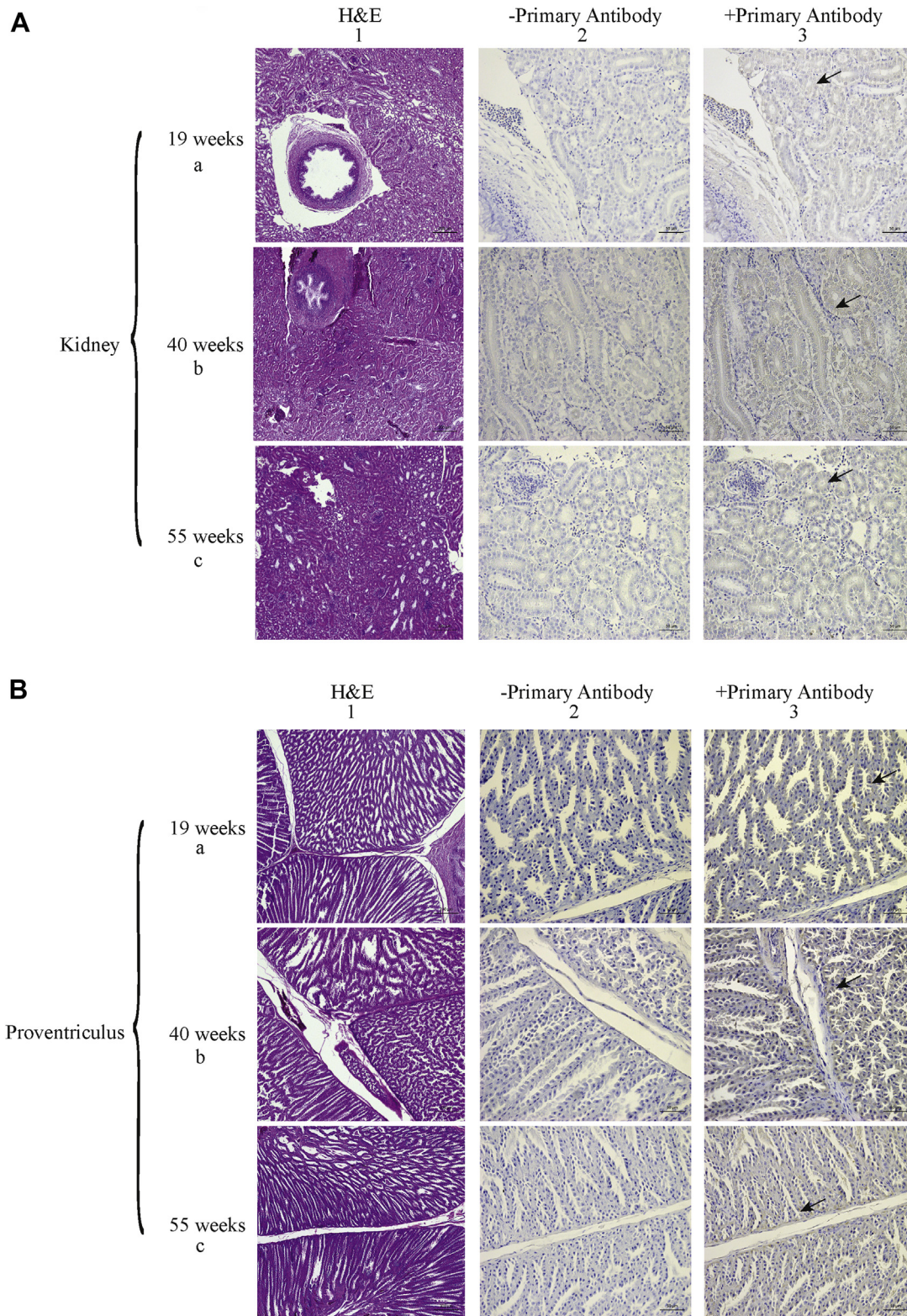


Figure 7. Haematoxylin and Eosin (H&E) staining (1) and chicken calcium-sensing receptor (cCaSR) immunohistochemistry (2, 3) in the kidney (A), proventriculus (B), duodenum (C), jejunum (D), ileum (E), colon (F), cecum (G), shell gland (H), and tibia (I) at 19 wk (a), 40 wk (b), and 55 wk (c) of age. Chicken CaSR immunoreactions are indicated as brown staining (+Primary Antibody, black arrows) and they are in contrast to blue counterstaining of the nuclei (-Primary Antibody). Medullary bone in the tibia is indicated in red circles. Scale bar for H&E staining represents 200 μ m, and for cCaSR immunohistochemistry is 50 μ m. Black arrows indicate positive staining.

calcium absorption through calcium transporters, with about 90% of calcium being absorbed in the small intestine (Bronner, 2009) explaining why cVDR is mainly expressed in the small intestine. When $1,25(\text{OH})_2\text{D}_3$ diffuses into cells, it binds to either cytoplasmic or nuclear

VDR and then triggers the formation of a heterodimer of VDR with a partner protein that binds to vitamin D response element (VDRE) located in the promoter region of target genes. VDRE has been found in a number of genes in chickens including $25(\text{OH})\text{D}_3$ 24-

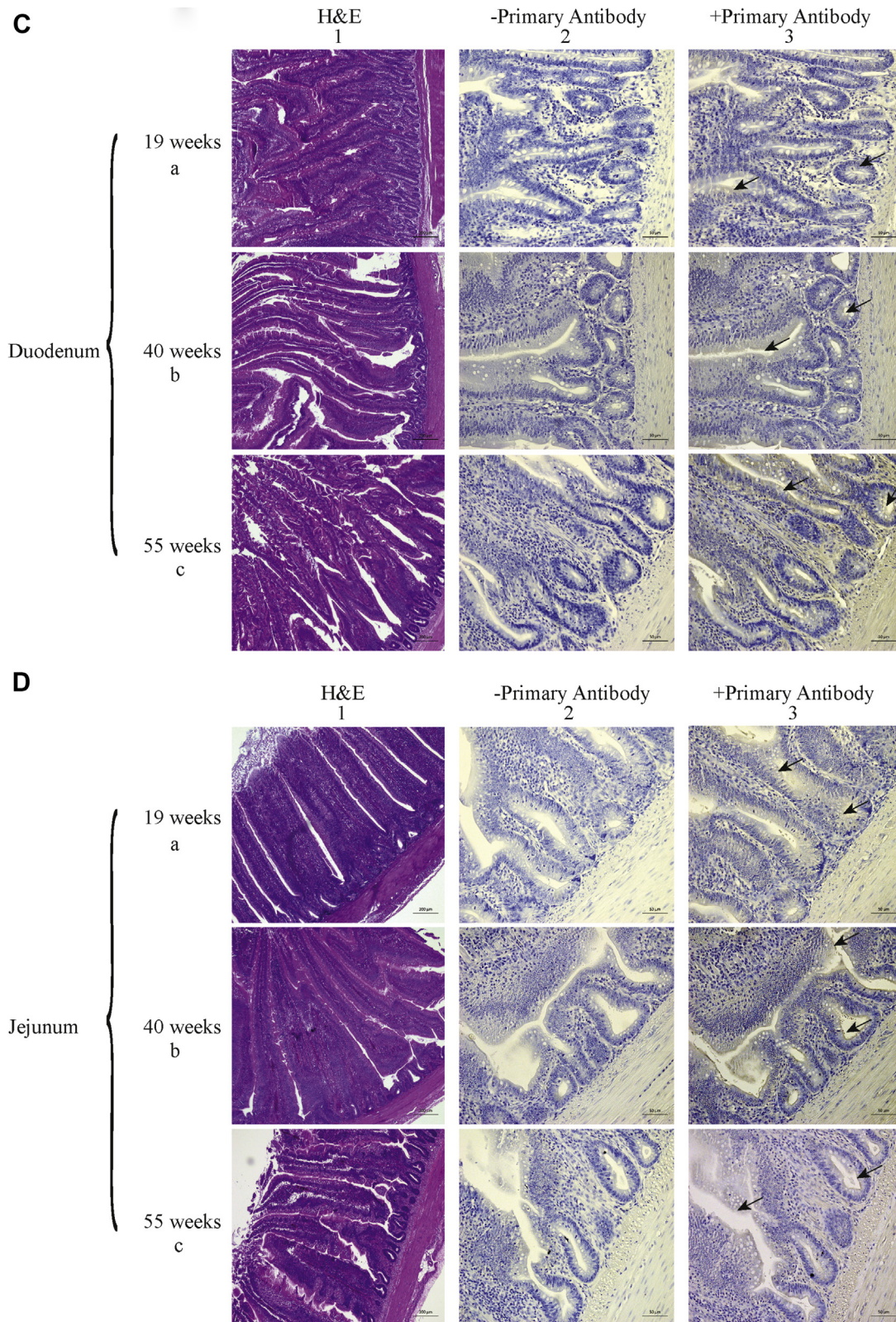


Figure 7. Continued

hydroxylase, PTH, calbindin D_{9k} and D_{28k} , calcium ion channels, CaSR, and osteocalcin, which are related to calcium absorption and homeostasis (Lu et al., 2000; Hendy and Canaff, 2016). However, during the process of calcium absorption in the small intestine, VDR is not the only element to respond to $1,25(\text{OH})_2\text{D}_3$.

Alternatively, $1,25(\text{OH})_2\text{D}_3$ only takes minutes to hours to exert effects via binding to the other protein without going through longer genomic pathways (Huhtakangas et al., 2004). Therefore, other possibilities should be considered regarding the regulation of calcium absorption and homeostasis in layers.

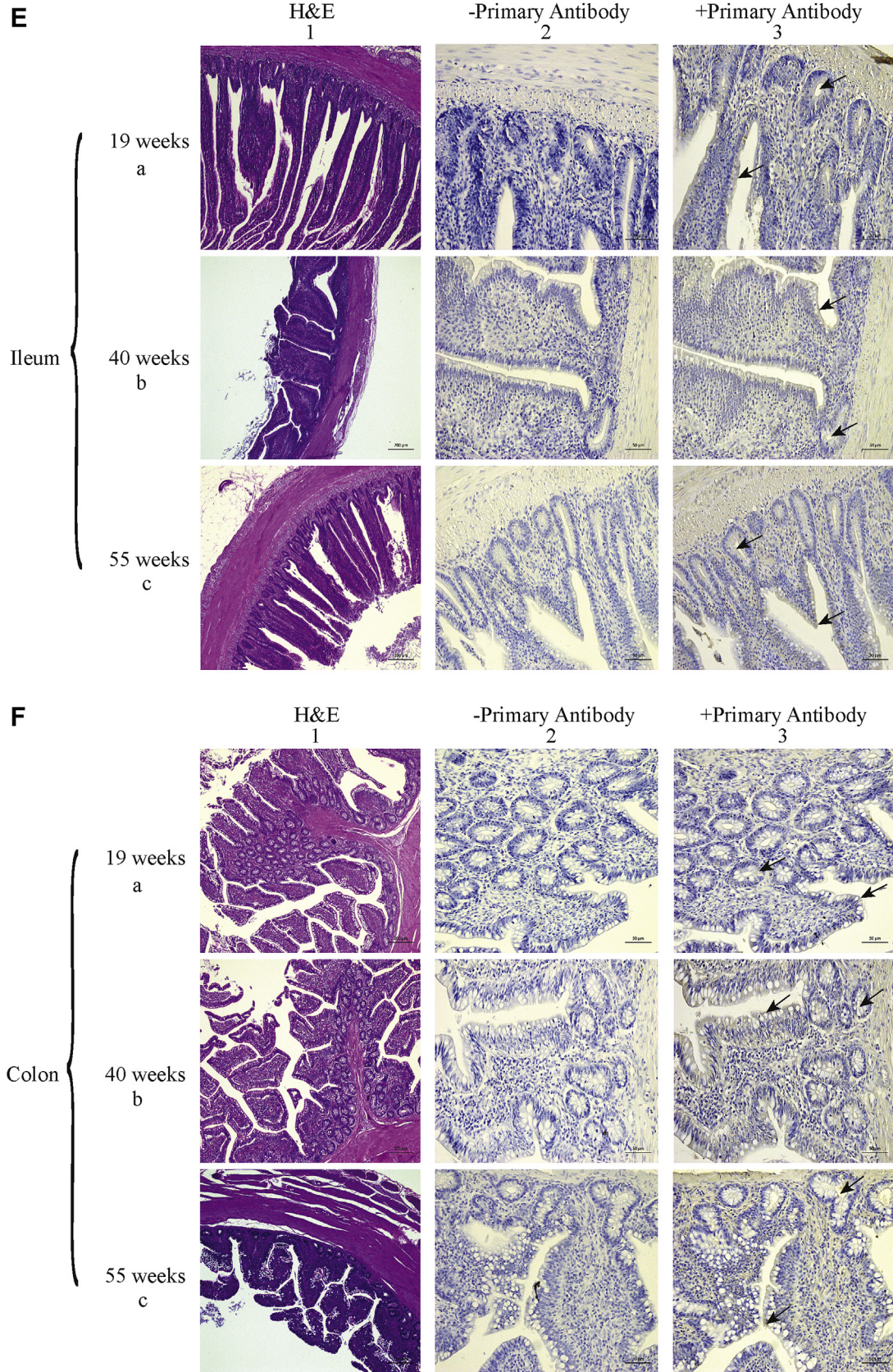


Figure 7. Continued

Nevertheless, the expression of cCaSR did not vary significantly in different tissues at peak production (40 wk of age). Western blot images showed that

CaSR had different forms in chickens. As the cCaSR exhibits a high homology and a similar molecular weight to mammal CaSR, the different forms of cCaSR might have

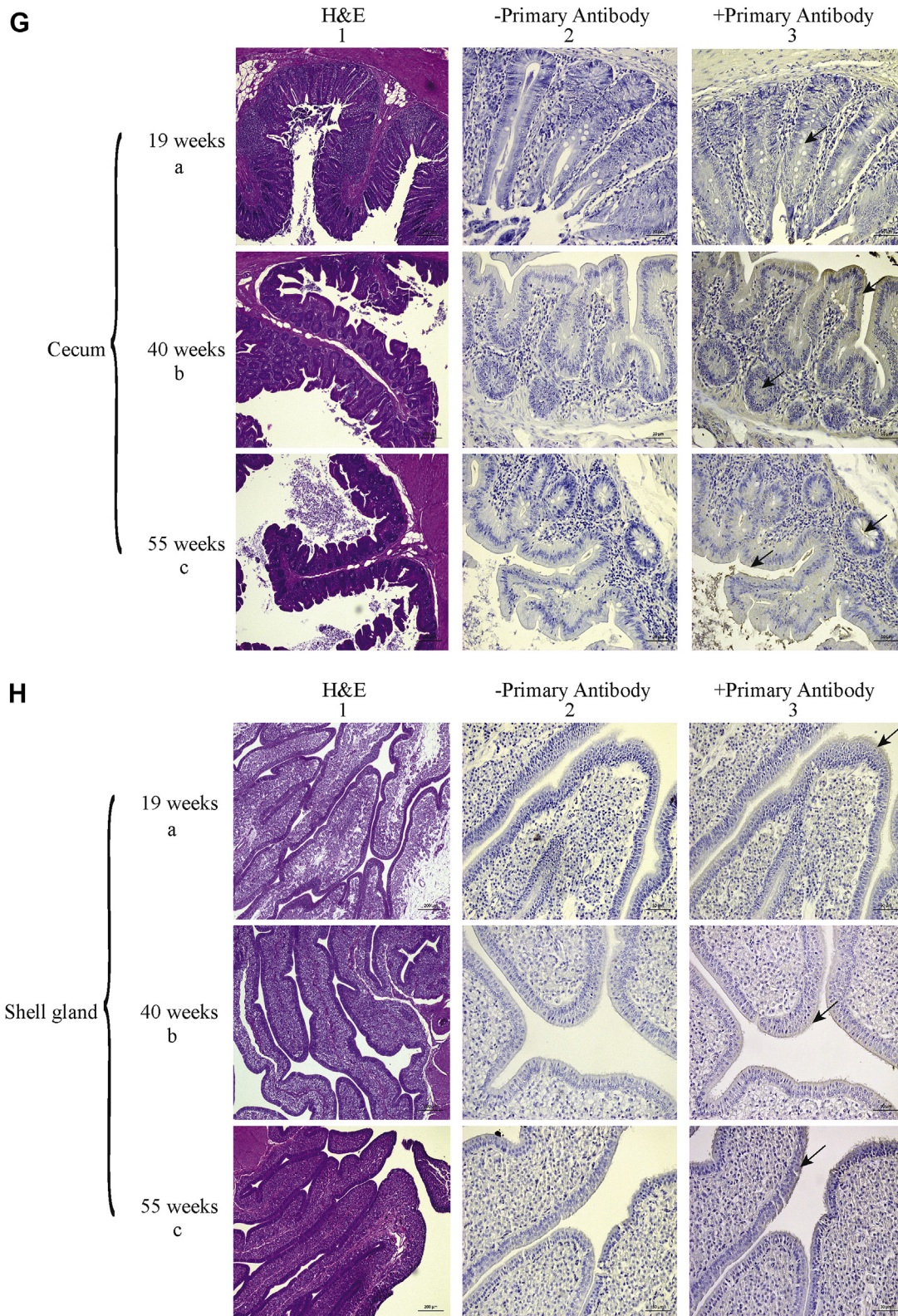


Figure 7. Continued

similar modifications. Therefore, in this study, approximately 120 kDa band might be unglycosylated cCaSR protein; approximately 140 kDa band might be the core-glycosylated, high-mannose form of cCaSR (in the endoplasmic reticulum); approximately 160 kDa might be maturely glycosylated form of cCaSR (in the Golgi

complex); more than 250 kDa band might be dimers and higher-order oligomers of cCaSR (White et al., 2009). In different tissues, we also observed that different forms of CaSR are expressed in different quantities. Various modifications and rapid turnover of CaSR probably resulted in a stable total amount of cCaSR.

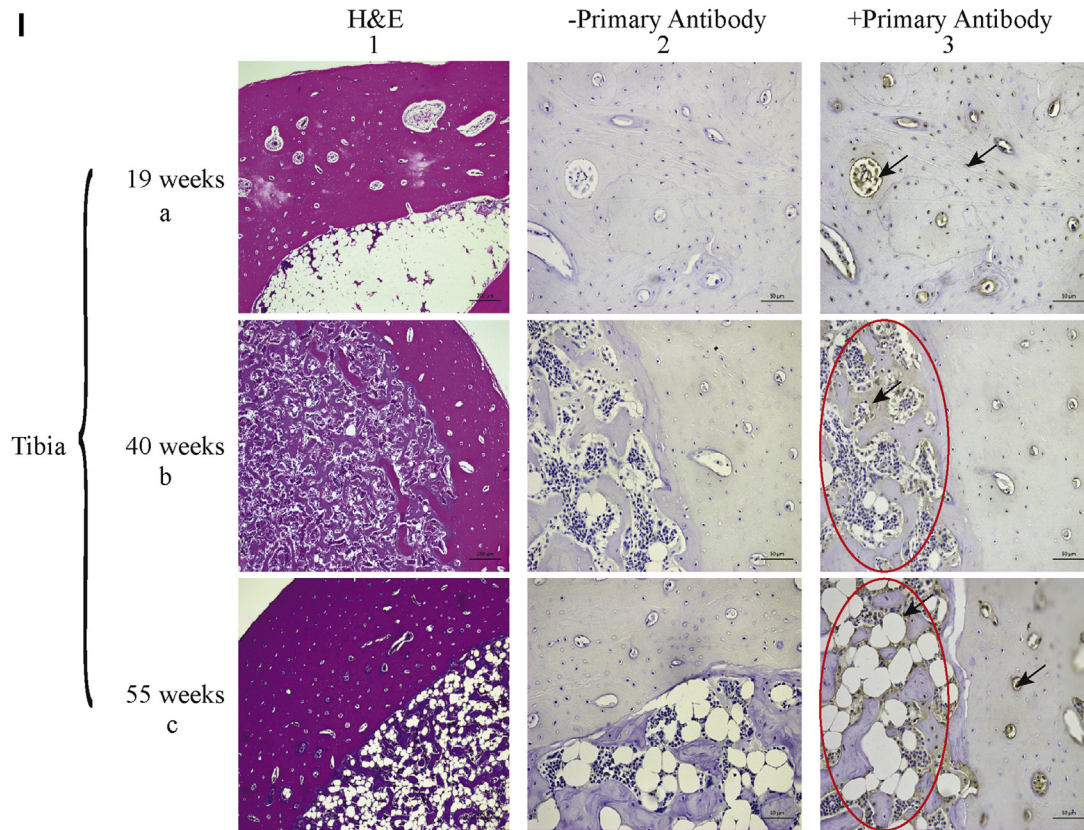


Figure 7. Continued

Calcium is absorbed by both an active transcellular pathway and a passive paracellular pathway through tight junctions. Most calcium transport proteins involved in the active transcellular pathway are $1,25(\text{OH})_2\text{D}_3$ and VDR-dependent. Among the small intestine, the duodenum has a highly active transport system (Christakos et al., 2011). That might be the reason why the expression of VDR in the duodenum is higher than that in other segments of the small intestine at peak production. Interestingly, different laying stages rarely affected the expression of both cCaSR and cVDR, which illustrated the importance of cCaSR and cVDR to regulate calcium homeostasis throughout the whole laying period.

The cCaSR was expressed in the nephron in the kidney including proximal tubules and glomerular structures. Intracellular calcium mobilization is an essential way for renal cells to respond to environmental stimulus and to achieve its function via CaSR involved in G_q -coupled inositol triphosphate signaling pathway or G_i -coupled cAMP changes (Magno et al., 2011), which is a potential reason why cCaSR was widely distributed in various structures of the kidney in laying hens. In addition, the GIT is another major site where cCaSR is located. The positive staining was found along the epithelium of the small intestine, which was consistent with what has been reported in mammals (Chattopadhyay et al., 1998; Zhao et al., 2019). It has been reported that CaSR is present in goblet cells and

enteroendocrine cells (Hira et al., 2008). Although enteroendocrine cells account for $\sim 1\%$ of epithelial cells, they play an important role in hormone secretion to communicate with other cells and relay signals to the brain, thus regulating gut function and metabolism (Liou, 2013). Therefore, it is of significance that a large number of GPCRs such as CaSR and taste receptors located in enteroendocrine cells contribute to nutrient sensing. Notably, the structure of female avian bone tissue is distinctive from mammals, which includes cortical, cancellous, and medullary bones (Kim et al., 2007). From morphological results of the tibia tissue, medullary bone was only found in the sexually matured layer (40- and 55-week-old) instead of the 19-week-old layer, which was consistent with previous research (Van de Velde et al., 1985). Medullary bone is a highly labile secondary bone, providing 35 to 40% of calcium for eggshell formation by its resorption to release minerals (Kim et al., 2012). It has been reported that hormones (estrogen and androgen) regulate the formation of medullary bones, but the growth of medullary bone causes structural bone (cortical bone) loss (Fisher and Schraer, 1982). Medullary bone resorption is a crucial biological process to release calcium, which is undertaken by osteoclasts. It has been reported that CaSR is expressed in osteoclasts. Activation of osteoclasts may result in the proliferation, differentiation, and apoptosis of osteoclasts (Dvorak et al., 2004; Mentaverri et al., 2006), indicating that CaSR might be an important receptor to

regulate bone resorption and maintain calcium homeostasis in laying hens.

In conclusion, this study demonstrated that cCaSR and cVDR were widely distributed in the kidney, proventriculus, duodenum, jejunum, ileum, colon, cecum, shell gland, and tibia of laying hens at different laying stages, especially cCaSR in the proventriculus, shell gland, and tibia, and its distribution pattern in the GIT, which was reported for the first time. Relative expression of cCaSR and cVDR varied in different tissues but did not change significantly among different laying stages. In addition, the localization in different tissues and the crystal structure of cCaSR was also highly similar to mammalian CaSR, which was evidence to support that cCaSR could be a potential therapeutic target to osteoporosis in laying hens.

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DISCLOSURE

There is no conflict interest for the authors to declare.

REFERENCES

- Abukawa, H., H. Mano, T. Arakawa, Y. Hakeda, H. Kimura, and M. Kumegawa. 2001. Tissue specific expression and differential regulation by 1α , 25-dihydroxyvitamin D₃ of the calcium-sensing receptor (CaSR) gene in rat kidney, intestine, and calvaria. *Cytotechnology* 35:81–86.
- Al-Batshan, H., S. Scheideler, B. Black, J. Garlich, and K. Anderson. 1994. Duodenal calcium uptake, femur ash, and eggshell quality decline with age and increase following molt. *Poult. Sci.* 73:1590–1596.
- Al-Dujaili, S. A., A. J. Koh, M. Dang, X. Mi, W. Chang, P. X. Ma, and L. K. McCauley. 2016. Calcium sensing receptor function supports osteoblast survival and acts as a co-factor in PTH anabolic actions in bone. *J. Cell. Biochem.* 117:1556–1567.
- Bar, A. 2009. Calcium transport in strongly calcifying laying birds: mechanisms and regulation. *Comp. Biochem. Phys. A.* 152:447–469.
- Berry, J., C. Farquharson, C. Whitehead, and E. Mawer. 1996. Growth plate chondrocyte vitamin D receptor number and affinity are reduced in avian tibial dyschondroplastic lesions. *Bone* 19:197–203.
- Bronner, F. 2009. Recent developments in intestinal calcium absorption. *Nutr. Rev.* 67:109–113.
- Brown, E. M., G. Gamba, D. Riccardi, M. Lombardi, R. Butters, O. Kifor, A. Sun, M. A. Hediger, J. Lytton, and S. C. Hebert. 1993. Cloning and characterization of an extracellular Ca²⁺-sensing receptor from bovine parathyroid. *Nature* 366:575–580.
- Brown, E. M., and R. J. MacLeod. 2001. Extracellular calcium sensing and extracellular calcium signaling. *Physiol. Rev.* 81:239–297.
- CCAC. 2009. Guidelines on the Care and Use of Farm Animals in Research, Teaching and Testing. Canadian Council on Animal Care, Ottawa, ON, Canada.
- Chattopadhyay, N., I. Cheng, K. Rogers, D. Riccardi, A. Hall, R. Diaz, S. C. Hebert, D. I. Soybel, and E. M. Brown. 1998. Identification and localization of extracellular Ca²⁺-sensing receptor in rat intestine. *Am. J. Physiol.-Gastroint. Liver Physiol.* 274:G122–G130.
- Cheng, I., I. Qureshi, N. Chattopadhyay, A. Qureshi, R. R. Butters, A. E. Hall, R. R. Cima, K. V. Rogers, S. C. Hebert, and J. P. Geibel. 1999. Expression of an extracellular calcium-sensing receptor in rat stomach. *Gastroenterology* 116:118–126.
- Christakos, S., P. Dhawan, A. Porta, L. J. Mady, and T. Seth. 2011. Vitamin D and intestinal calcium absorption. *Mol. Cell. Endocrinol.* 347:25–29.
- Conigrave, A. D. 2016. The calcium-sensing receptor and the parathyroid: past, present, future. *Front. Physiol.* 7:563.
- Cransberg, P., G. Parkinson, S. Wilson, and B. Thorp. 2001. Sequential studies of skeletal calcium reserves and structural bone volume in a commercial layer flock. *Br. Poult. Sci.* 42:260–265.
- Cufadar, Y., O. Olgun, and A. Yildiz. 2011. The effect of dietary calcium concentration and particle size on performance, eggshell quality, bone mechanical properties and tibia mineral contents in moulted laying hens. *Br. Poult. Sci.* 52:761–768.
- Deng, G., J. Bi, J. Qiao, G. He, B. Wu, M. Zhang, and N. Lv. 2010. Expression and tissue distribution of extracellular calcium-sensing receptor (CaSR) mRNA in chickens. *Turk. J. Vet. Anim. Sci.* 34:249–254.
- Diaz, R., S. Hurwitz, N. Chattopadhyay, M. Pines, Y. Yang, O. Kifor, M. S. Einat, R. Butters, S. C. Hebert, and E. Brown. 1997. Cloning, expression, and tissue localization of the calcium-sensing receptor in chicken (*Gallus domesticus*). *Am. J. Physiol.-Regul. Integr. Comp. Physiol.* 273:R1008–R1016.
- Dvorak, M. M., A. Siddiqua, D. T. Ward, D. H. Carter, S. L. Dallas, E. F. Nemeth, and D. Riccardi. 2004. Physiological changes in extracellular calcium concentration directly control osteoblast function in the absence of calciotropic hormones. *Proc. Natl. Acad. Sci. USA* 101:5140–5145.
- Fisher, L. W., and H. Schraer. 1982. Keratan sulfate proteoglycan isolated from the estrogen-induced medullary bone in Japanese quail. *Comp. Biochem. Phys. B.* 72:227–232.
- Fleming, R. H. 2008. Nutritional factors affecting poultry bone health: symposium on ‘Diet and bone health’. *Proc. Nutr. Soc.* 67:177–183.
- Fudge, N. J., and C. S. Kovacs. 2004. Physiological studies in heterozygous calcium sensing receptor (CaSR) gene-ablated mice confirm that the CaSR regulates calcitonin release *in vivo*. *BMC Physiol* 4:5.
- Ganjigohari, S., N. Ziaei, A. Ramzani Ghara, and S. Tasharofi. 2018. Effects of nanocalcium carbonate on egg production performance and plasma calcium of laying hens. *J. Anim. Physiol. Anim. Nutr.* 102:e225–e232.
- Geng, Y., L. Mosyak, I. Kurinov, H. Zuo, E. Sturchler, T. C. Cheng, P. Subramanyam, A. P. Brown, S. C. Brennan, and H.-c. Mun. 2016. Structural mechanism of ligand activation in human calcium-sensing receptor. *eLife* 5:e13662.
- Halse, J., S. Greenspan, F. Cosman, G. Ellis, A. Santora, A. Leung, N. Heyden, S. Samanta, S. Doleckyj, and E. Rosenberg. 2014. A phase 2, randomized, placebo-controlled, dose-ranging study of the calcium-sensing receptor antagonist MK-5442 in the treatment of postmenopausal women with osteoporosis. *J. Clin. Endocrinol. Metab.* 99:E2207–E2215.
- Hebert, S. C. 1996. Extracellular calcium-sensing receptor: implications for calcium and magnesium handling in the kidney. *Kidney Int.* 50:2129–2139.
- Hebert, S. C., S. Cheng, and J. Geibel. 2004. Functions and roles of the extracellular Ca²⁺-sensing receptor in the gastrointestinal tract. *Cell Calcium* 35:239–247.
- Hendy, G. N., and L. Canaff. 2016. Calcium-sensing receptor gene: regulation of expression. *Front. Physiol.* 7:394.
- Hira, T., S. Nakajima, Y. Eto, and H. Hara. 2008. Calcium-sensing receptor mediates phenylalanine-induced cholecystokinin secretion in enteroendocrine STC-1 cells. *FEBS J.* 275:4620–4626.
- Huhtakangas, J. A., C. J. Olivera, J. E. Bishop, L. P. Zanello, and A. W. Norman. 2004. The vitamin D receptor is present in caveolae-enriched plasma membranes and binds 1α , 25 (OH)₂-vitamin D₃ *in vivo* and *in vitro*. *Mol. Endocrinol.* 18:2660–2671.
- Hurwitz, S., and A. Bar. 1966. Rate of passage of calcium-45 and Yttrium-91 along the intestine, and calcium absorption in the laying Fowl. *J. Nutr.* 89:311–316.

- Jensen, A. A., and H. Brauner-Osborne. 2007. Allosteric modulation of the calcium-sensing receptor. *Curr. Neuropharmacol.* 5:180–186.
- Keshavarz, K. 2003. A comparison between cholecalciferol and 25-OH-cholecalciferol on performance and eggshell quality of hens fed different levels of calcium and phosphorus. *Poult. Sci.* 82:1415–1422.
- Kim, W. K., S. A. Bloomfield, T. Sugiyama, and S. C. Ricke. 2012. Concepts and methods for understanding bone metabolism in laying hens. *Worlds Poult. Sci. J.* 68:71–82.
- Kim, W., L. Donalson, S. Bloomfield, H. Hogan, L. Kubena, D. Nisbet, and S. Ricke. 2007. Molt performance and bone density of cortical, medullary, and cancellous bone in laying hens during feed restriction or alfalfa-based feed molt. *Poult. Sci.* 86:1821–1830.
- Knowles, T., and L. Wilkins. 1998. The problem of broken bones during the handling of laying hens—a review. *Poult. Sci.* 77:1798–1802.
- Kos, C. H., A. C. Karaplis, J.-B. Peng, M. A. Hediger, D. Goltzman, K. S. Mohammad, T. A. Guise, and M. R. Pollak. 2003. The calcium-sensing receptor is required for normal calcium homeostasis independent of parathyroid hormone. *J. Clin. Invest.* 111:1021–1028.
- Liang, G.-B., C. Zhou, X. Huo, H. Wang, X. Yang, S. Huang, H. Wang, H. Wilkinson, L. Luo, and W. Tang. 2016. Discovery of novel dihydrobenzofuran cyclopropane carboxylic acid based calcium sensing receptor antagonists for the treatment of osteoporosis. *Bioorg. Med. Chem. Lett.* 26:4077–4080.
- Liou, A. 2013. Digestive physiology of the pig symposium: G protein-coupled receptors in nutrient chemosensation and gastrointestinal hormone secretion. *J. Anim. Sci.* 91:1946–1956.
- Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25:402–408.
- Lu, Z., K. Hanson, and H. F. DeLuca. 1997. Cloning and origin of the two forms of chicken vitamin D receptor. *Arch. Biochem. Biophys.* 339:99–106.
- Lu, Z., F. Jehan, C. Zierold, and H. F. DeLuca. 2000. Isolation and characterization of the chicken vitamin D receptor gene and its promoter. *J. Cell. Biochem* 77:92–102.
- Magno, A. L., B. K. Ward, and T. Ratajczak. 2011. The calcium-sensing receptor: a molecular Perspective. *Endocr. Rev.* 32:3–30.
- Mentaverri, R., S. Yano, N. Chattopadhyay, L. Petit, O. Kifor, S. Kamel, E. Terwilliger, M. Brazier, E. Brown, and R. Mentaverri. 2006. The calcium sensing receptor is directly involved in both osteoclast differentiation and apoptosis. *FASEB J.* 20:2562–2564.
- Olgun, O., and A. Aygun. 2016. Nutritional factors affecting the breaking strength of bone in laying hens. *Worlds Poult. Sci. J.* 72:821–832.
- Proszkowiec-Weglarczyk, M., and R. Angel. 2013. Calcium and phosphorus metabolism in broilers: effect of homeostatic mechanism on calcium and phosphorus digestibility. *J. Appl. Poult. Res.* 22:609–627.
- Quarles, L. D. 2003. Extracellular calcium-sensing receptors in the parathyroid gland, kidney, and other tissues. *Curr. Opin. Nephrol. Hypertens.* 12:349–355.
- Rath, N., G. Huff, W. Huff, and J. Balog. 2000. Factors regulating bone maturity and strength in poultry. *Poult. Sci.* 79:1024–1032.
- Riccardi, D., and E. M. Brown. 2010. Physiology and pathophysiology of the calcium-sensing receptor in the kidney. *Am. J. Physiol.-Renal Physiol.* 298:F485–F499.
- Riccardi, D., J. Park, W. S. Lee, G. Gamba, E. M. Brown, and S. C. Hebert. 1995. Cloning and functional expression of a rat kidney extracellular calcium/polyvalent cation-sensing receptor. *Proc. Natl. Acad. Sci. USA* 92:131–135.
- Roura, E., I. Depoortere, and M. Navarro. 2019. Chemosensing of nutrients and non-nutrients in the human and porcine gastrointestinal tract. *Animal* 13:2714–2726.
- Van de Velde, J., J. Vermeiden, and A. Bloot. 1985. Medullary bone matrix formation, mineralization, and remodeling related to the daily egg-laying cycle of Japanese quail: a histological and radiological study. *Bone* 6:321–327.
- White, E., J. McKenna, A. Cavanaugh, and G. E. Breitwieser. 2009. Pharmacochaperone-mediated rescue of calcium-sensing receptor loss-of-function mutants. *Mol. Endocrinol.* 23:1115–1123.
- Whitehead, C., and R. Fleming. 2000. Osteoporosis in cage layers. *Poult. Sci.* 79:1033–1041.
- Yarden, N., I. Lavelin, O. Genina, S. Hurwitz, R. Diaz, E. M. Brown, and M. Pines. 2000. Expression of calcium-sensing receptor gene by avian parathyroid gland *in vivo*: relationship to plasma calcium. *Gen. Comp. Endocrinol.* 117:173–181.
- Yoshimura, Y., H. Ohira, and T. Tamura. 1997. Immunocytochemical localization of vitamin D receptors in the shell gland of immature, laying, and molting hens. *Gen. Comp. Endocrinol.* 108:282–289.
- Zhao, X., B. Schindell, W. Li, L. Ni, S. Liu, C. U. Wijerathne, J. Gong, C. M. Nyachoti, K. O., and C. Yang. 2019. Distribution and localization of porcine calcium sensing receptor in different tissues of weaned piglets. *J. Anim. Sci.* 97:2402–2413.