Comparison of sex determination mechanism of germ cells between birds and fish: Cloning and expression analyses of chicken forkhead box L3-like gene

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Abstract

Background: Birds harbor specific sex determination and differentiation mechanisms. Although the molecular mechanisms associated with sex determination in somatic cells have been elucidated, those for germ cells remain unclear.

Results: Here, we characterized the chicken forkhead box L3 (foxl3)-like gene as a sex-determination factor in sexually indifferent medaka germline stem cells. The foxl3-like gene was cloned by rapid amplification of cDNA ends, and the nucleotide sequence was analyzed. The deduced amino acid sequence was compared with FOXL3 sequences from other species, revealing low identity and similarity scores. Expression analysis of foxl3-like mRNA during gonadogenesis showed female left-gonad-specific temporal expression in an egg incubated from 10 to 16 days, as well as low general expression in certain hatched female chicken organs. Moreover, the amino acid sequence deduced for the FOXL3-like protein displayed low identity with medaka FOXL3, with the FOXL3-like protein specifically localized in the oogonia, whereas medaka FOXL3 was found in sexually indifferent germline stem cells. Furthermore, the timing of expression differed between the foxl3-like gene and that of medaka foxl3.

Conclusions: These results suggest that chicken FOXL3-like protein and medaka FOXL3 differ in terms of their functions as female sex-determination factors.

KEYWORDS
chick embryo, feminization, foxl3-like gene, gonadogenesis

1 | INTRODUCTION

Avian sex-determination and -differentiation mechanisms exhibit characteristic features. The development of the urogenital system in chickens (Gallus gallus) resembles that of mammals. Contrasting, sex hormones have significant effects on sex determination and differentiation in chickens, with the mechanisms similar to those in fish. For example, Taber reviewed a phenomenon observed by Aristotle (384–322 BC), describing female-to-male sex reversal in chickens. Moreover, a previous study has reported the induction of masculinization via inhibition of aromatase in a female chicken embryo. These findings suggest that birds exhibit specific sex-determination and -differentiation mechanisms, with characteristics resembling those seen in mammals and fish. Therefore, elucidation of these mechanisms in birds is important for understanding vertebrate evolution.

Previous reports revealed chicken-specific sex-determination and -differentiation mechanisms in somatic cells. Doublesex and mab-3 related transcription factor 1 (dmrt1) is a key molecular factor in avian gonad masculinization, and dmrt1 knockout
in male chicken embryos induce gonadal feminization, down-regulation of a testicular differentiation marker (SRY-box 9; sox9), and ectopic expression of female markers (aromatase and forkhead box L2; foxl2). Furthermore, dmrt1 overexpression induces male pathway genes in female embryonic gonads. These results suggest dmrt1 as a candidate master gene involved in avian testis determination. Chicken hemogen (cHEMGN) is a species-specific factor involved in gonadal masculinization, and after dmrt1 expression, cHEMGN is expressed in pre-Sertoli cells. Additionally, chicken somatic cells possess a cell-autonomous sex-determination process. Notably, these molecular mechanisms have not been observed in other species.

Although the sex of somatic cells has been investigated, the molecular mechanisms associated with germ-cell sex determination have not yet been analyzed. To elucidate the general mechanism involved in sex-determination and -differentiation in chickens, it is necessary to investigate the decision process associated with germ-cell fate.

In the present study, we targeted the forkhead box L3 (foxl3) gene. In medaka (Oryzias latipes), foxl3/FOXNL is expressed in both male and female germline stem cells and associated with sperm-egg fate decision. Additionally, FOXL3 levels in germline stem cells is down-regulated by the dmrt1 gene has not yet been cloned and characterized in chickens; therefore, we investigated the role of the chicken foxl3 gene in the decision-making process involved with germ-cell fate in order to offer further insight into the evolution of the associated molecular mechanism.

Here, we cloned chicken foxl3-like gene cDNA by rapid amplification of cDNA ends (RACE) and obtained the complete nucleotide sequence. The predicted chicken FOXL3-like protein amino acid sequence was compared with that of FOXL3 from other species, including medaka, and we analyzed the dynamics of chicken foxl3-like gene transcription during embryogenesis by quantitative real-time (q)PCR. Additionally, we investigated the expression of the chicken foxl3-like gene in somatic tissues of 2-week-old female chickens and determined the localization of the FOXL3-like protein by immunofluorescence staining.

2 RESULTS

2.1 Sequence analysis

To clone chicken foxl3-like gene cDNA fragments, specific primers were used for PCR and 3' and 5' RACE. A schematic diagram of chicken foxl3-like gene shows the primers used and the positions of the fragments obtained in this study (Figure 1).

Fragment 1 was initially obtained by qRT-PCR with primers a and b which were designed from the predicted chicken foxl3 sequence (Galgal4:21:3447683:3448402:1). The nucleotide sequence was analyzed and fragment 1 was found to be 513 bp long. The 5'-RACE revealed the nucleotide sequence of the 5' end of chicken foxl3-like gene. GeneRacer 5' primer (primer c) and primer d (based on the nucleotide sequence of fragment 1) were used in this method. We obtained fragment 2 by 5'-RACE and found that it was 252 bp long. We also analyzed the sequence of the 3' end of chicken foxl3-like gene by 3' RACE using primer e and GeneRacer 3' primer (primer f); fragment 3 was obtained and its length was found to be 611 bp.

The chicken foxl3-like mRNA sequence, including the 3' and 5' untranslated regions (UTRs), was determined from the sequences of fragments 1, 2, and 3 (Figure 2). The length of the open reading frame (ORF) was 762 bp encoding 254 amino acids, with the predicted size of the forkhead domain (a DNA-binding domain conserved forkhead box family) was 96 amino acids. The ORF displayed a GC content of 76.2%, and the ATG initiation and TAG termination codons were localized at nucleotides 27–29 and 789–791, respectively. The 3' UTR contained a poly (A) tail. While the poly (A) signal motif

![FIGURE 1 Schematic of chicken foxl3-like gene cDNA cloning. Primers targeting the chicken foxl3-like gene sequence. The chicken foxl3-like ORF (open box) and the forkhead DNA-binding domain (black box) are shown. Primer sites are designated by arrows](image-url)
AATAAA was absent, a similar sequence, TATAAA, was detected at nucleotides 1224–1229.

The sequence data reported in this article have been submitted to DDBJ/EMBL/Genbank (accession no. LC455571).

2.2 Amino acid sequence homology between chicken FOXL3-like protein and FOXL3 from other species

We compared the chicken FOXL3-like protein sequence with FOXL3 from the zebra finch (Taeniopygia guttata), gray short-tailed opossum (Monodelphis domestica), and medaka (Figure 3).

The lengths of the chicken FOXL3-like protein and that from the zebra finch, opossum, and medaka comprised 254, 246, 283, and 263 amino acids, respectively, with the N-terminal region of the chicken FOXL3-like protein the shortest among the species. The forkhead domain (46 amino acids) was highly conserved among all four species, whereas there was minimal shared identity outside of this domain, as only two amino acids corresponded among the four species.

We then determined identity and similarity scores between the FOXL3-like protein and FOXL2 encoded by forkhead-box family genes conserved among all vertebrates and involved in gonadal feminization (Table 1). The amino acid sequence homology between chicken FOXL3-like protein and FOXL3 from other species:Chicken FOXL3-like protein sequence and forkhead DNA-binding domain (black boxes) are shown. The termination codon is marked with three asterisks, and a polyA-like signal sequence (TATAAA) is underlined.
The acid sequence of chicken FOXL3-like protein shared 40.9% to 48.3% identity and 57.3% to 60.2% similarity with FOXL2 from other species. Additionally, the forkhead domain of the chicken FOXL3-like protein showed a sequence identity and similarity of 58.9% to 71.9% and 84.2% to 85.4% with the same domain in FOXL3 from other species, respectively. Relative to the identity and similarity scores for FOXL2, those of FOXL3-like protein were low. Therefore, conservation of the FOXL3 sequence was low among vertebrates.

To analyze the relationship between the chicken FOXL3-like protein and sequences of other FOXL family proteins, a phylogenetic tree was constructed using the neighbor-joining method in MEGA7 (https://www.megasoftware.net/; Figure 4), with confidence values generated by bootstrapping based on 1000 resampling replicates. The results indicated that the chicken FOXL3-like protein belonged to the FOXL family and was evolutionarily closer to the FOXL2 family than the FOXL1 family.

### Table 1

| Amino acid identity (similarity) (%) | Mature protein | Forkhead domain |
|------------------------------------|----------------|-----------------|
| **Chicken**                        | **Zebra finch** | **Opossum**     | **Medaka**     |
| FOXL3-like protein                 | 48.3 (60.2)     | 42.5 (57.8)     | 40.9 (57.3)    |
|                                   | 71.9 (85.4)     | 67.3 (84.2)     | 58.9 (84.2)    |
| **FOXL2**                          | 91.9 (98.0)     | 66.6 (74.9)     | 80.1 (91.5)    |
|                                   | 100 (100)       | 98.9 (100)      | 98.9 (100)     |

The results of qPCR for chicken foxl3-like mRNA-expression profiles during embryogenesis. Chicken foxl3-like gene expression was detected in the female left gonad in an egg incubated for 10–16 days (Figure 5). In birds, the male and female gonads develop symmetrically and asymmetrically, respectively, with both the male right and left gonads becoming functional testes, whereas only the left gonad becomes a functional ovary in the female, and the female right gonad regresses and nearly disappears before hatching. In the male right and left gonads, chicken foxl3-like mRNA-expression levels were consistently low in an egg incubated for 4.5–20 days, whereas mRNA levels were up-regulated in the female left gonad of an egg incubated for 8 days. Additionally, temporal expression was observed in the gonad in an egg incubated for 8–18 days; this was also observed in the female right gonad, although at lower levels than those in the female left gonad.

### Expression analysis in various tissues of post-hatched chickens

Using qPCR, we determined chicken foxl3-like mRNA-expression levels in the ovary, heart, bursa of Fabricius,
intestine, kidney, spleen, liver, brain, and thymus harvested from 2-week-old chickens (Figure 6A). The results showed significantly lower expression in all of the aforementioned tissues than that observed in the female left gonad in an egg incubated for 14 days. Additionally, the foxl3-like gene was highly expressed in the liver, although its expression score was approximately 24-fold lower than that in the female left gonad in an egg incubated for 14 days. Moreover, expression level in the ovaries of 2-week-old female chickens was approximately 103-fold lower than that in the female left gonad in an egg incubated for 14 days. Investigation of foxl3-like mRNA expression in the testes of 2-week-old male chickens revealed low levels of expression similar to those in ovaries from 2-week-old female chickens (Figure 6B).

2.5 Immunofluorescence staining

To determine localization of the chicken FOXL3-like protein, immunofluorescence staining was performed on a female left gonad in an egg incubated for 14 days. The specific anti-FOXL3-like antibodies used in this analysis were characterized by immunofluorescence staining in HEK293 cells transfected with recombinant chicken FOXL3-like protein (Figure 7A,B). Since a previous study reported that medaka FOXL3 was expressed in germine stem cells, we hypothesized that the chicken FOXL3-like protein would also be expressed in germ cells. To test this hypothesis, we used a monoclonal antibody against chicken vasa homolog (CVH), a chicken germ-cell-specific molecule, along with the anti-FOX3-like protein antibody. Total IgG purified from non-immunized serum with anti-CVH antibody was used as a negative control. The results showed that CVH- and FOXL3-like-protein-positive cells were localized in the nuclei (Figure 7C-H). Germ cells in the female left gonad at this stage represent oogonia, which showed CVH-positive staining in the region of the cortex of the gonad. These results suggested that chicken FOXL3-like protein localized to the nuclei of oogonia. Additionally, we determined localization of the chicken FOXL3-like protein in an ovary from a mature female chicken and a left gonad from a male embryo in an egg incubated for 14 days; however, we detected no chicken FOXL3-like protein-positive staining in either tissues (data not shown).

3 DISCUSSION

Chicken sex-determination and differentiation mechanisms exhibit characteristics resembling those of both mammals and fish. Therefore, it is useful to establish avian sex-determination and differentiation mechanisms to elucidate the evolutionary processes associated with sex determination in vertebrates. However, in avian germ cells, these molecular mechanisms are poorly understood. The foxl3 gene is involved in sex determination in medaka germ cells; therefore, in the present study, we identified a chicken foxl3-like gene and characterized its role in germ cells from chickens relative to that in medaka germ cells.

The amino acid sequence of the ORF in chicken FOXL3-like protein was compared with those of the FOXL in other species. The forkhead domain sequence was found to be highly conserved in four different species (Figure 3). However, the identity and similarity scores for the entire protein sequence across the four species were lower than those for the FOXL2 sequence (Table 1). This suggested that the FOXL3 amino acid sequence changed rapidly during vertebrate evolution. This finding was consistent with results from previous studies showing an absence of foxl3 in...
amphibians and placental mammals. Moreover, construction of a phylogenetic tree revealed that the chicken foxl3-like gene belongs to the FOXL3 subset of the FOXL family (Figure 4). FOXL2 is related to ovarian development in mammals and teleosts, and in chickens, foxl2 mRNA is highly expressed in female gonads in eggs incubated for 5.7 days, with the timing of expression similar to that of the aromatase gene. These findings suggest that chicken FOXL2 and that from other vertebrates are associated with gonadal feminization in the early embryo. Furthermore, a previous study showed that foxl2 and foxl3 are ancient paralogs in teleosts, which suggests that chicken foxl2 and the foxl3-like gene are likely also ancient paralogs. A recent report characterizing foxl3 in Japanese eel (Anguilla japonica) revealed the presence of two foxl3 genes (foxl3a and foxl3b) in this species. In the present study, investigation of a possible foxl3 parologue in chickens revealed no results according to NCBI BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of current genome databases using chicken FOXL3-like amino acid sequence (our unpublished data).

We investigated the timing of chicken foxl3-like mRNA expression in both male and female gonads during embryogenesis (Figure 5), and identified temporal up-regulation only in the female gonad in an egg incubated for 8–18 days. Additionally, the timing of chicken foxl3-like gene expression differed from that of medaka foxl3, which is expressed in both male and female germ cells at the same developmental stage. In medaka, FOXL3 levels were down-regulated in surrounding testicular somatic cells, thereby displaying temporal expression and inducing spermatogenesis. By contrast, we did not observe up-regulation of chicken foxl3-like gene expression in the male gonad. This difference suggests that the function of the chicken FOXL3-like protein differs from that of medaka FOXL3, and that the germ-cell sex-determination mechanism and/or sex-determination-related factors in chickens might differ from those in medaka.

We determined localization of the chicken FOXL3-like protein in female chicken gonads using CVH-positive cells in an egg incubated for 14 days (Figure 7). The results indicated that chicken FOXL3-like protein was expressed in the oogonia, suggesting that the protein functions as a germ-cell intrinsic factor during feminization. In chickens, migration of primordial germ cells to the future gonadal region (intermediate mesoderm) via the blastoderm was first observed in an egg incubated for 54 hours. Moreover, differentiation of a sexually undifferentiated gonad into a testis or ovary

![FIGURE 5 Analysis of chicken foxl3-like gene expression during embryogenesis. Real-time PCR analysis of chicken foxl3-like gene expression in female and male left-right gonads during embryogenesis. Three to eight embryos were used at each stage of embryogenesis. The female right gonads in eggs incubated for 18–20 days were too small to be harvested. The average scores are represented by lines. The 2^−ΔΔCt method was used to compare expression levels, which were normalized to β-actin. The score of the male right gonads in eggs incubated for 4.5 days was used as a calibrator. Error bars indicate the SE of the mean, and significance was evaluated between male and female gonads on the same incubation days and gonad locations. *P < .05; **P < .01; ***P < .005; ****P < .001.](https://example.com/figure5)
was observed in an egg incubated for approximately 6–6.5 days. Furthermore, the sox9 gene, which is normally expressed in the developing vertebrate testis, was observed to be expressed in the male gonad in an egg incubated for 6.0 days. On the other hand, estrogen is required for ovary development in a female chicken embryo. Aromatase participates in estrogen synthesis and was detected only in the female gonad in an egg incubated for 6.0 days, and is up-regulated during ovary development. Male and female germ cells display different proliferative activity. During sex differentiation of the male gonad, there is virtually no germ-cell proliferation, whereas female germ cells in the left ovarian cortex start to undergo mitosis as early as 9 days into egg incubation. Moreover, the number of germ cells increases ~25-fold at between 9 and 17 days of incubation. In an egg incubated for 15.5 days, meiosis is initiated only in female germ cells. In the present study, we found that in the female left gonad, expression of the chicken foxl3-like gene was up-regulated starting on day 8 and down-regulated after 16 days of incubation. These data suggested that the chicken FOXL3-like protein functions during the oogonial-proliferation stage and then ceases to function at the onset of meiosis.

Previous studies elucidated a chicken-specific sex-determination mechanism in somatic cells. For example, cHEMGN, a homolog specifically expressed in hematopoietic mouse tissues, participates in the early sex-determination stage in chickens. Moreover, another study identified a cell-autonomous sex-determination process in chickens. In the present study, we showed that functions associated with the chicken foxl3-like gene differed from that of the medaka foxl3, and that the FOXL3-mediated germ-cell sex-determination mechanism observed in medaka is not conserved in chickens. Therefore, chickens might exhibit a unique germ-cell-producing mechanism and sex-determination system.

4 | EXPERIMENTAL PROCEDURES

4.1 | Animals and tissues

Gonads from an embryo and ovary, and the heart, bursa of Fabricius, intestine, kidney, spleen, liver, brain, and thymus from 2-week-old female chickens were harvested from White Leghorn chickens (Akita, Japan). Testes of 2-week-old male Plymouth Rock chickens were harvested (Okazaki, Japan). The chickens were kept in an isolation facility at the University Animal Farm, Hiroshima University, Japan, and maintained, bred, and used in experiments in accordance with Hiroshima University guidelines. The study protocol was approved by the Experimental Animal Committee of Hiroshima University (Authorization No. C16-23-2).

4.2 | RT-PCR to clone the chicken foxl3-like gene

Total RNA was extracted from the gonads of a 2-week-old chicken using TRIzol reagent and reverse transcribed with SuperScript III reverse transcriptase (Thermo Fisher Scientific, Waltham, Massachusetts) according to manufacturer instructions. To amplify and analyze the chicken foxl3-like gene cDNA sequence, a primer pair (primers a and b) was designed from the predicted chicken foxl3 sequence (Galgal4:21:3447683:3448402:1), with primer a based on the forkhead box domain. The primer a and b sequences were 5'-GCCTCAACCCCTGCTCTTCC-3' and 5'-CCGGCTCGGGCGGCACTGG-3', respectively. KOD FX (Toyobo Co. Ltd., Osaka, Japan) was used as a DNA polymerase for PC
R amplification of the chicken foxl3-like gene, which was performed on a SimpliAmp Thermal Cycler (Thermo Fisher Scientific) under the following conditions: 35 cycles of 98°C for 10 seconds and 68°C for 30 seconds. Products were separated by electrophoresis on 3% agarose LO3 gels (TaKaRa Bio, Shiga, Japan) and visualized with ethidium bromide. For nested
PCR using the same reagents and conditions, two oligo primers (primers a2 and b2) were used: 5'-CGGCGGCGAATTGGGC TCTGGAC-3' and 5'-CGGGCTCGAAGTGGGGCGAAGC -3', respectively.

4.3 Cloning and sequencing

The chicken foxl3-like gene cDNA fragment derived from PCR with primers a and b was purified using a QIAquick PCR Purification kit (Qiagen, Hilden, Germany) according to manufacturer's instructions, and purified fragments were inserted into a pCR2.1-TOPO vector using a Topo TA Cloning kit (Invitrogen, Carlsbad, California) for cloning. The nucleotide sequences were analyzed with M13 reverse and forward primers using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, California).

The 5' end of the chicken foxl3-like gene was obtained by 5' RACE using a GeneRacer Kit (Thermo Fisher Scientific). RNA oligo (GeneRacer RNA oligo) was ligated to the 5' end of the chicken foxl3-like mRNA according to manufacturer instructions. Total RNA was reverse transcribed with PrimeScript reverse transcriptase (TaKaRa Bio). Three primer pairs were used to amplify the 5' end of chicken foxl3-like gene cDNA. For the first amplification, 5'-GSP1, 5'-AGACATGGCCAGCCGTAACGGCA (primers a2 and b2) were used: 5'-GGAGCGGCGGAGGGAGATT-3' and GeneRacer 3'primers were used. For the second amplification, primers e, 5'-AGATCGGCTTCGCCCCACTGC-3' and f, 5'-CGCTACGTAACGGCAGTGACGC-3' were used. Step-down PCR and sequence analysis were performed as described above.

4.4 Real-time PCR (qPCR)

The expression of chicken foxl3-like mRNA was determined by qPCR. Total RNA was isolated using an RNeasy Mini Kit (Qiagen). To eliminate genomic DNA, total RNA was treated with RNase-free DNase (Qiagen). cDNA synthesis was then performed with PrimeScript reverse transcriptase (TaKaRa Bio), and qPCR was performed using a StepOne real-time PCR system (Applied Biosystems) with KOD SYBR qPCR mix (Toyobo Co. Ltd.). Primers a and 5'-GSP1 served as forward and reverse primers, respectively. Real-time qPCR was performed under the following conditions: 50 cycles of 98°C for 10 seconds and 68°C for 40 seconds. Chicken foxl3-like mRNA expression was evaluated by the 2^-ΔΔCt method,26 and scores for each threshold cycle (Ct) were normalized against those for β-actin. To amplify β-actin fragments, two primers were used: forward, 5'-AATCCGGAACC TCCATTGTGC-3' and reverse, 5'-GCCATGGCAATCTCGTC TTG-3'. Significance was evaluated by a two-tailed Student's t-test assuming unequal variance.

4.5 Sex discrimination in chicken embryos

The sexes of chicken embryos in eggs incubated for 10–20 days were determined according to gonadogenesis patterns (male gonads develop symmetrically, whereas female gonads develop asymmetrically). The sexes of embryos in eggs incubated for 4.5–8 days were established according to the patterns of chromodomain-helicase DNA-binding protein-1 fragment bands obtained by PCR.27,28 Genomic DNA was extracted from small embryonic tissue fragments with a Puregene core kit A (Qiagen) according to manufacturer instructions.

4.6 Antibody production and immunofluorescence staining

The anti-CVH mouse monoclonal antibody29 and anti-chicken FOXL3-like protein rabbit polyclonal antibody were used for this assay. The anti-chicken FOXL3-like protein polyclonal antibody was generated by immunizing rabbits with peptide corresponding to the C-terminal region (PEPGSRRPLASSR; amino acids 236–248) at a dilution of 1:50. The specificity of the anti-chicken FOXL3-like protein antibody was established by immunofluorescence staining in
HEK293 cells transfected with a pcDNA 3.1/myc-His A vector (Invitrogen) harboring the cloned ORF encoding the chicken FOXL3-like protein. Primers F and R were designed to amplify the chicken FOXL3-like protein ORF: 5'-CCCGAATTCCCGGCGATGCGGGACGAG-3' and 5'-CCGCTCGAGGGCAGCACCAGGCCTGC-3', respectively. The PCR conditions were the same as those described. Hybridoma supernatant containing anti-CVH and anti-chicken FOXL3-like protein antibodies was used for the primary antibody reaction at 1:2 and 1:50 dilutions, respectively. In the secondary antibody reaction, the highly cross-adsorbed secondary antibodies goat anti-mouse IgG (H+L) (Alexa Fluor Plus 488; Invitrogen) and goat anti-rabbit IgG (H+L) (Alexa Fluor 594; Invitrogen) at 1:200 dilution were used to detect CVH and chicken FOXL3-like protein, respectively.

Gonads were fixed in 4% paraformaldehyde in phosphate-buffered saline overnight, and after rinsing with water, the samples were dehydrated with an ethanol series (70%, 80%, 90%, 95%, 99%, and 100%), immersed in xylene, and embedded in paraffin. Sections were cut into 4-μm slices using a microtome (SM2000R; Leica Biosystems, Wetzlar, Germany), and stained with hematoxylin and eosin. For antigen activation, the sections were deparaffinized, hydrated, heated in 10 mM sodium citrate buffer (pH 6.0) at 110°C for 20 minutes, and washed in Tris-buffered saline with Tween-20 (TBST; 25 mM Tris-HCl, 2.5 mM KCl, 150 mM NaCl, and 0.1% Tween-20 (pH 7.7)). They were then blocked in 2% skim milk for 1 hour at room temperature, after which the primary antibody reaction was performed in 2% skim milk-TBST at 4°C overnight in a humidified chamber. The sections were washed in TBST, and the secondary antibody reaction was performed for 1 hour at room temperature, followed by washing in TBST and nuclear staining with 4',6-diamidino-2-phenylindole (DAPI) in Vectashield Mounting Medium (Vector Laboratories, Burlingame, California). The negative control used for chicken FOXL3-like protein detection comprised sections exposed to non-immunized serum with the anti-CVH antibody. All sections were observed under a fluorescence microscope (BX51; Olympus, Tokyo, Japan) and photographed with an Olympus DP70 camera (Olympus).

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