PATTERNS & PHENOTYPES

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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. Contrastingly, 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.[1] Moreover, a previous study has reported the induction of masculinization via inhibition of aromatase in a female chicken embryo.[2] 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* knockdown

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*).[3] Furthermore, *dmrt1* overexpression induces male pathway genes in female embryonic gonads.[4] 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,[5] and after *dmrt1* expression, *cHEMGN* is expressed in pre-Sertoli cells. Additionally, chicken somatic cells possess a cell-autonomous sex-determination process.[6] 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*/FOXL3 is expressed in both male and female germline stem cells and associated with sperm-egg fate decision.[7,8] Additionally, FOXL3 levels in germline stem cells is down-regulated by spermatogenesis induced by testicular somatic cells, and functional sperm cells have been observed in the ovaries of *foxl3*^{−/−} female medaka. The *foxl3* gene is conserved in all vertebrates, except amphibians and placental mammals,[9-11] but absent in mice (*Mus musculus*), in which the mechanism of deciding germ-cell fate differs from that of medaka. However, the *foxl3* 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
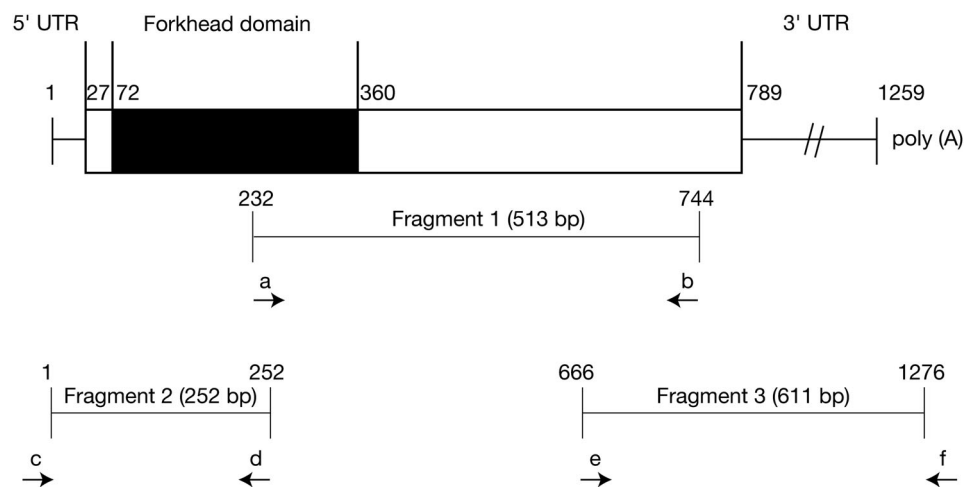

FIGURE 1 Schematic of chicken *foxl3*-like gene cDNA cloning. Primers targeting the chicken *foxl3*-like gene sequence. The chicken *foxl3*-like ORF (open box) and the forkhead DNA-binding domain (black box) are shown. Primer sites are designated by arrows

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
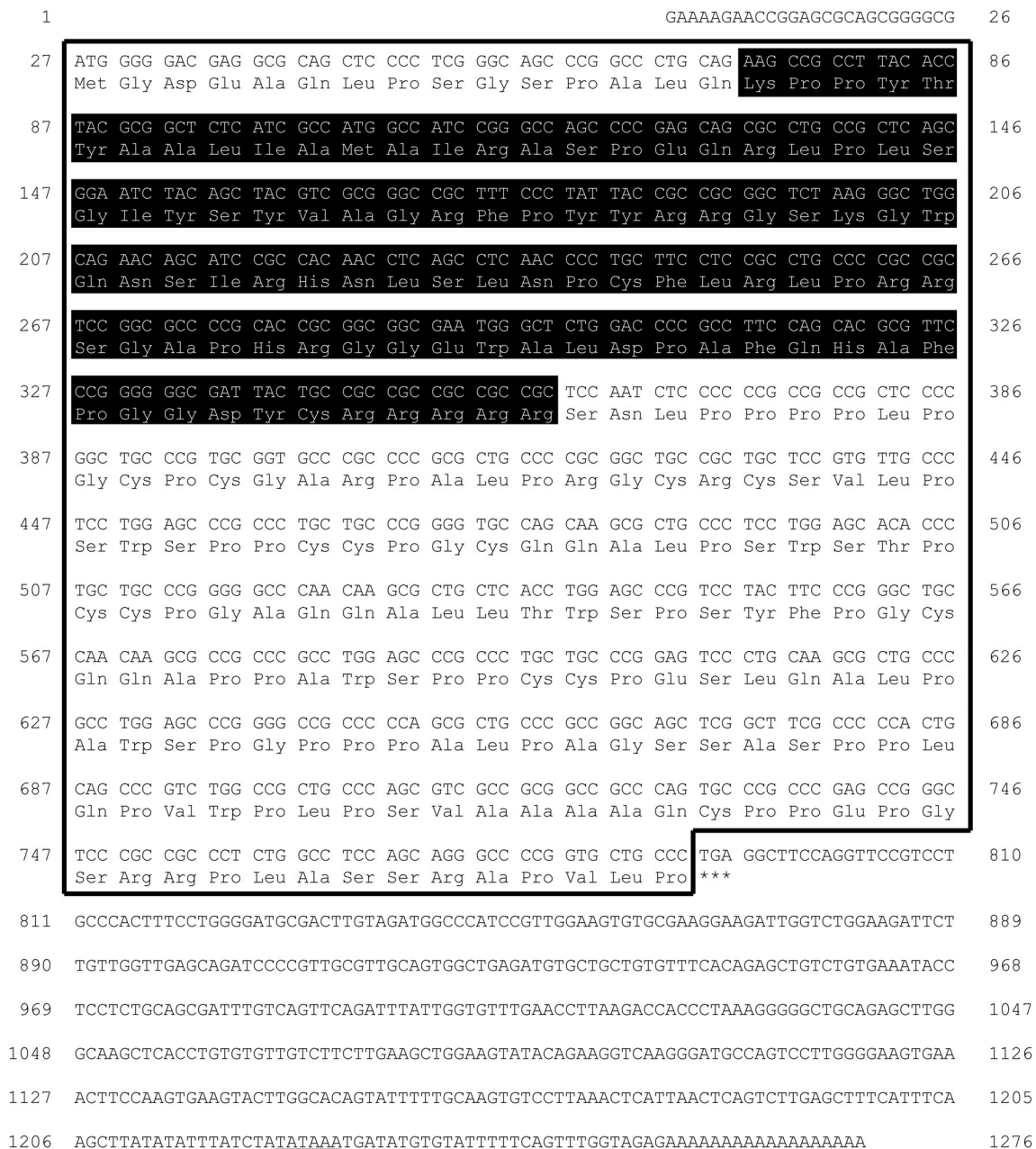
```
   1                                                    GAAAAGAACCGGAGCGCAGCGGGGCG   26

  27   ATG GGG GAC GAG GCG CAG CTC CCC TCG GGC AGC CCG GCC CTG CAG AAG CCG CCT TAC ACC   86
       Met Gly Asp Glu Ala Gln Leu Pro Ser Gly Ser Pro Ala Leu Gln Lys Pro Pro Tyr Thr

  87   TAC GCG GCT CTC ATC GCC ATG GCC ATC CGG GCC AGC CCC GAG CAG CGC CTG CCG CTC AGC  146
       Tyr Ala Ala Leu Ile Ala Met Ala Ile Arg Ala Ser Pro Glu Gln Arg Leu Pro Leu Ser

 147   GGA ATC TAC AGC TAC GTC GCG GGC CGC TTT CCC TAT TAC CGC CGC GGC TCT AAG GGC TGG  206
       Gly Ile Tyr Ser Tyr Val Ala Gly Arg Phe Pro Tyr Tyr Arg Arg Gly Ser Lys Gly Trp

 207   CAG AAC AGC ATC CGC CAC AAC CTC AGC CTC AAC CCC TGC TTC CTC CGC CTG CCC CGC CGC  266
       Gln Asn Ser Ile Arg His Asn Leu Ser Leu Asn Pro Cys Phe Leu Arg Leu Pro Arg Arg

 267   TCC GGC GCC CCG CAC CGC GGC GGC GAA TGG GCT CTG GAC CCC GCC TTC CAG CAC GCG TTC  326
       Ser Gly Ala Pro His Arg Gly Gly Glu Trp Ala Leu Asp Pro Ala Phe Gln His Ala Phe

 327   CCG GGG GGC GAT TAC TGC CGC CGC CGC CGC CGC TCC AAT CTC CCC CCG CCG CCG CTC CCC  386
       Pro Gly Gly Asp Tyr Cys Arg Arg Arg Arg Arg Ser Asn Leu Pro Pro Pro Pro Leu Pro

 387   GGC TGC CCG TGC GGT GCC CGC CCC GCG CTG CCC CGC GGC TGC CGC TGC TCC GTG TTG CCC  446
       Gly Cys Pro Cys Gly Ala Arg Pro Ala Leu Pro Arg Gly Cys Arg Cys Ser Val Leu Pro

 447   TCC TGG AGC CCG CCC TGC TGC CCG GGG TGC CAG CAA GCG CTG CCC TCC TGG AGC ACA CCC  506
       Ser Trp Ser Pro Pro Cys Cys Pro Gly Cys Gln Gln Ala Leu Pro Ser Trp Ser Thr Pro

 507   TGC TGC CCG GGG GCC CAA CAA GCG CTG CTC ACC TGG AGC CCG TCC TAC TTC CCG GGC TGC  566
       Cys Cys Pro Gly Ala Gln Gln Ala Leu Leu Thr Trp Ser Pro Ser Tyr Phe Pro Gly Cys

 567   CAA CAA GCG CCG CCC GCC TGG AGC CCG CCC TGC TGC CCG GAG TCC CTG CAA GCG CTG CCC  626
       Gln Gln Ala Pro Pro Ala Trp Ser Pro Pro Cys Cys Pro Glu Ser Leu Gln Ala Leu Pro

 627   GCC TGG AGC CCG GGG CCG CCC CCA GCG CTG CCC GCC GGC AGC TCG GCT TCG CCC CCA CTG  686
       Ala Trp Ser Pro Gly Pro Pro Pro Ala Leu Pro Ala Gly Ser Ser Ala Ser Pro Pro Leu

 687   CAG CCC GTC TGG CCG CTG CCC AGC GTC GCC GCG GCC GCC CAG TGC CCG CCC GAG CCG GGC  746
       Gln Pro Val Trp Pro Leu Pro Ser Val Ala Ala Ala Ala Gln Cys Pro Pro Glu Pro Gly

 747   TCC CGC CGC CCT CTG GCC TCC AGC AGG GCC CCG GTG CTG CCC TGA GGCTTCCAGGTTCCGTCCT   810
       Ser Arg Arg Pro Leu Ala Ser Ser Arg Ala Pro Val Leu Pro ***

 811   GCCCACTTTCCTGGGGATGCGACTTGTAGATGGCCCATCCGTTGGAAGTGTGCGAAGGAAGATTGGTCTGGAAGATTCT   889

 890   TGTTGGTTGAGCAGATCCCCGTTGCGTTGCAGTGGCTGAGATGTGCTGCTGTGTTTCACAGAGCTGTCTGTGAAATACC   968

 969   TCCTCTGCAGCGATTTGTCAGTTCAGATTTATTGGTGTTTGAACCTTAAGACCACCCTAAAGGGGGCTGCAGAGCTTGG  1047

1048   GCAAGCTCACCTGTGTGTTGTCTTCTTGAAGCTGGAAGTATACAGAAGGTCAAGGGATGCCAGTCCTTGGGGAAGTGAA  1126

1127   ACTTCCAAGTGAAGTACTTGGCACAGTATTTTTGCAAGTGTCCTTAAACTCATTAACTCAGTCTTGAGCTTTCATTTCA  1205

1206   AGCTTATATATTTATCTATATAAATGATATGTGTATTTTTCAGTTTGGTAGAGAAAAAAAAAAAAAAAAAA          1276
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FIGURE 2 Nucleotide and deduced amino acid sequences. The chicken *foxl3*-like ORF (open box) and the 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

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chicken FOXL3-like protein   1:-----------------------MGDEAQLPSGSPALQ KPPYTYAALIAMAIRASPEQR  36
zebra finch FOXL3            1:--------MAETDPGERRAVGTERGTERGTGPGTAGWPQ RPPYSFVALITMAIRASPEQR  52
opossum FOXL3               1:MATQDFTAQPAPPQASPTEVAPGRGSGQLAGGPRPDPLQ KPPYSYVALIAMAIRESPEQR  60
medaka FOXL3               1:---------MDAEEKPIGERGVQLLDISSTPEEAPPLE KPPYSYVALIAMAIKDSRDQR  50
                                                        .   ...***...***.***. *...**

chicken FOXL3-like protein  37:LPLSGIYSYVAGRFPYYRRGSKGWQNSIRHNLSLNPCFLRLPRRS----GAPHRGGEWAL  92
zebra finch FOXL3           53:LSLSGIYAYIAERFPFYRGRGRQWQNSVRHNLSLNPCFRRLPGRH----G---RAGEWAL 105
opossum FOXL3              61:LPLSGIYRYIVARFPYYERNQKGWQNSVRHNLSLNECFVKVPRDGCPGASSERKGNLWAL 120
medaka FOXL3              51:KTLGGIYQYIISKFPYYEKNKKGWQNSIRHNLSLNECFVKVPREN----GGDKKGNFWML 106
                              . *.***  *.  .**.*  ..**** ******* **  *.    .   .   *.*

chicken FOXL3-like protein  93:DPAFQHAFPGGDYCRRRRR----SNLPPPPLPGCPCGARPALPRGCRCSVLPSWSPPCCP 148
zebra finch FOXL3          106:DPAFQDMFPGGNYLRRRRRLCRRPSASPPPPPGTPPGPAAAGPPPPSGIPPGPAEPPQLP 165
opossum FOXL3             121:DPAFRDMFDQGDYRRRRRRPRPRLRAPRPGPPPTGRSPAPLPPAFPKLSCCPLPPPQRPL 180
medaka FOXL3             107:DPACEDMFEKGNYRRRRR-----VR--RTYRPPSAPCTTGNPVEYPE----PLYHYLQPA 155
                              ***. ..* *  * * ****.      .  *             .

chicken FOXL3-like protein 149:GCQQA-LPSWSTPCCPGAQQ-ALLTWSPSYFPGCQQAPPAWSPPCCPESLQ-ALPAWSPG 205
zebra finch FOXL3          166:EIHPGSAAPWPPPAHPVPPGWPQGPWAALVLPPRYPELPVPGPPVSPGPPPLPLPAWALH 225
opossum FOXL3             181:YLLRDPWAPGQPQPGCAPSVGGPTGNSRAFSHSPGPCQPRSPGQ--PGSGYRAPAAAPTA 238
medaka FOXL3             156:YM-TNSWSLCAPGSSPQTAYPAPQVVSPQ-PRSLSPSGPFYPPHFFQHAVYGGHHRHPSV 213
                                                                .  *       .  .

chicken FOXL3-like protein 206:PPPALPAGSSASPPLQPVWPLPSVAAAAQCPPEPGSRRPLASSRAPVLP-           254
zebra finch FOXL3          226:PAELGTAVTCELGATLAPAFR---------------------------           246
opossum FOXL3             239:RLPHY-WGGAGAVQHLWSSQARRLTAEALPGFPPQLSSAPTAQLDF----           283
medaka FOXL3             214:LVPHNGWPYGGVTQPMCPDGGSAAVACGYQQLAPYGRQTESPALGFQSDP           263
                                        .                 .        .
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FIGURE 3 Comparison of the chicken FOXL3-like protein sequence with other species. Amino acids identified in the four species are marked with asterisks. Shared sequences common to three of the four species are marked with points. The sequence corresponding to the forkhead domain is indicated by boxes

TABLE 1 Identity and similarity scores of chicken FOXL3-like and FOXL2 compared with FOXL3 and FOXL2 of other species, respectively

| 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)

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.

2.3 | Expression analysis during embryogenesis

qPCR was performed to investigate 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.

2.4 | 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,
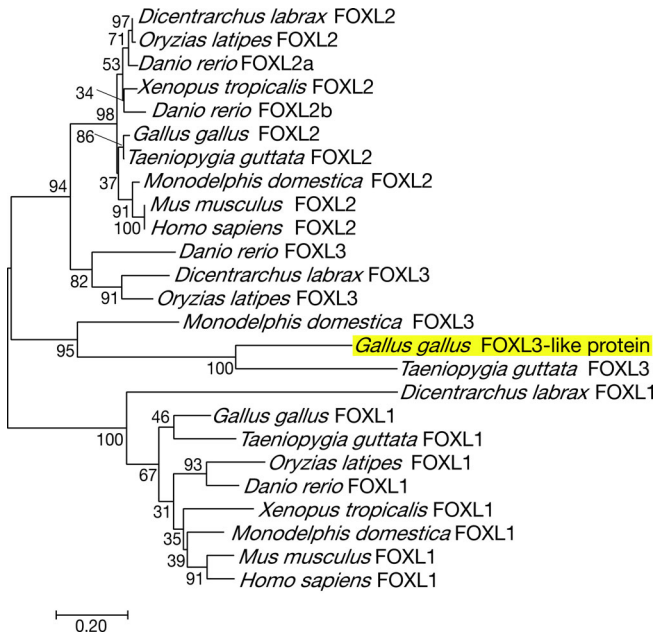
FIGURE 4 Phylogenetic tree FOXL family proteins in vertebrates. The tree was constructed by the neighbor-joining method in MEGA7. Percentages of the 1000-bootstrap test are shown next to the branches. The chicken FOXL3-like protein is highlighted. Accession numbers of the sequences used in this analysis are as follows: *D. labrax* (European bass) FOXL1 (CBN81215), FOXL2 (ACW83540), and FOXL3 (AFV13295); *D. rerio* (Zebrafish) FOXL1 (NP_957278), FOXL2a (NP_001038717), FOXL2b (NP_001304690), and FOXL3 (AAI62838); *G. gallus* (Chicken) FOXL1 (XP_001231599) and FOXL2 (NP_001012630); *H. sapiens* (Human) FOXL1 (NP_005241) and FOXL2 (NP_075555); *M. domestica* (Opossum) FOXL1 (XP_007477502), FOXL2 (XP_007494005), and FOXL3 (XP_007491677); *M. musculus* (Mouse) FOXL1 (NP_032050) and FOXL2 (NP_036150); *O. latipes* (Medaka) FOXL1 (NP_001116391), FOXL2 (NP_001098358), and FOXL3 (XP_004070713); *T. guttata* (Zebra finch) FOXL1 (XP_002194184), FOXL2 (XP_002187454), and FOXL3 (taeGut3.2.4:21:5092875:5093615:-1); and *X. tropicalis* (Tropical clawed frog) FOXL1 (AAI35134) and FOXL2 (XP_004917868)

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 germline 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,[12] along with the anti-FOXL3-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,[13] 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 FOXL3 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
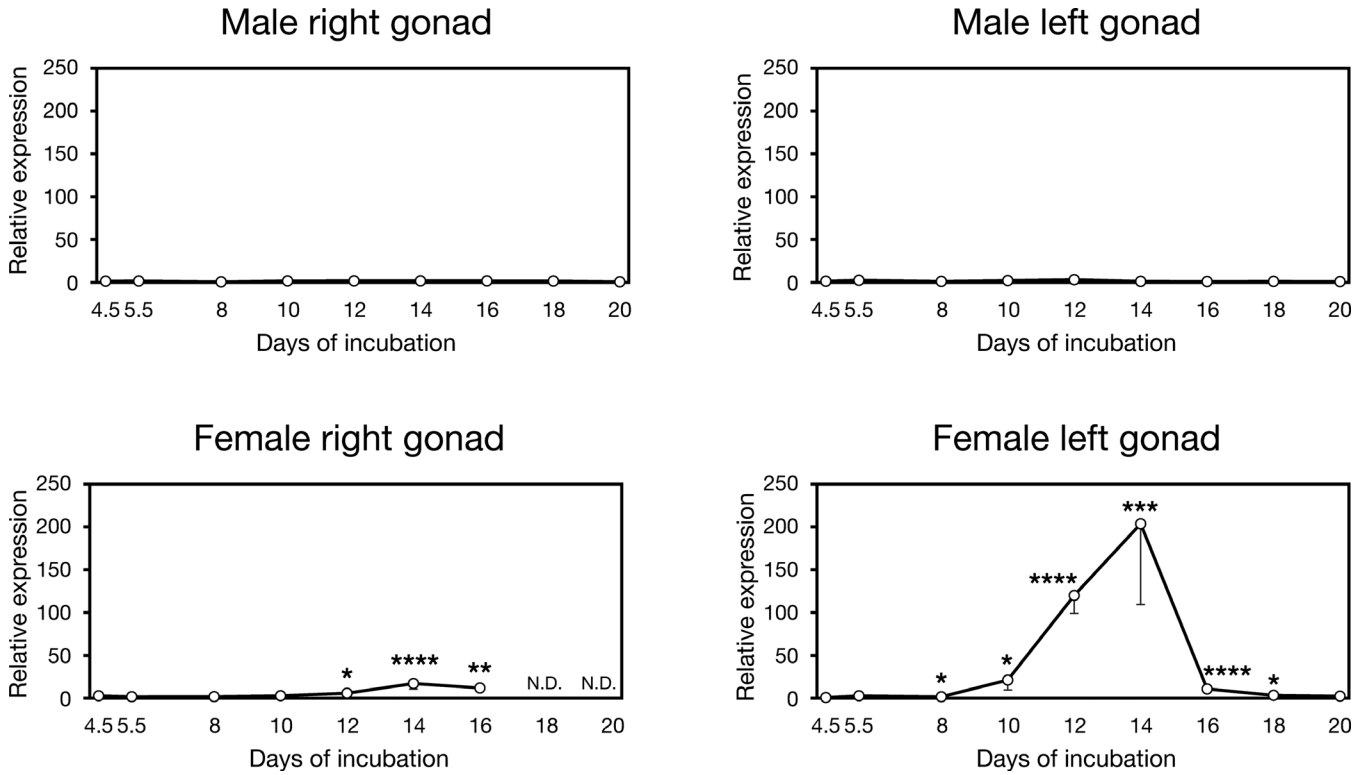
FIGURE 5 Analysis of chicken *foxl3*-like gene expression during embryogenesis. Real-time PCR analysis of chicken *foxl3*-like gene expression in female and male left-right gonads during embryogenesis. Three to eight embryos were used at each stage of embryogenesis. The female right gonads in eggs incubated for 18–20 days were too small to be harvested. The average scores are represented by lines. The $2^{-\Delta\Delta Ct}$ 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. *$P < .05$; **$P < .01$; ***$P < .005$; ****$P < .001$

amphibians and placental mammals.[9-11] 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[14] and teleosts,[15] 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.[16] 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,[10] 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.[17] In the present study, investigation of a possible *foxl3* paralogue 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.[7,8] In male 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.[18] Moreover, differentiation of a sexually undifferentiated gonad into a testis or ovary
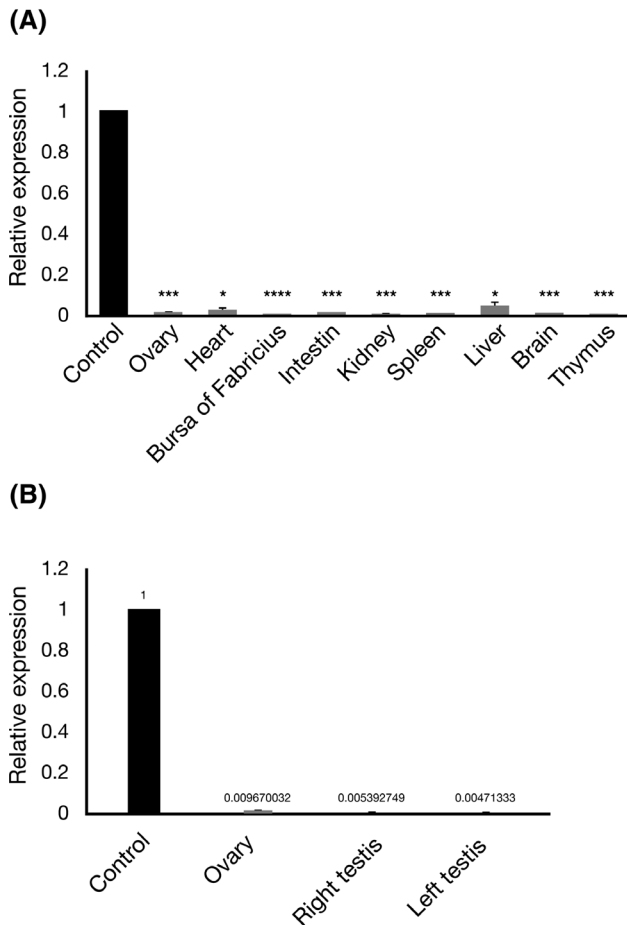
(A)

(B)

FIGURE 6 Analysis of chicken *foxl3*-like gene expression in various tissues. A, Real-time PCR analysis of chicken *foxl3*-like gene expression was performed in various tissues from 2-week-old female chickens (n = 3 samples/tissue) and normalized against β-actin levels using $2^{-\Delta\Delta Ct}$ method. Expression of the chicken *foxl3*-like gene in the female left gonad in eggs incubated for 14 days was used as a calibrator. Error bars indicate SE of the mean. B, Expression of the chicken *foxl3*-like gene in the ovary of 2-week-old female chickens was compared with that in the testes of 2-week-old male chickens following normalization against *β-actin* levels $2^{-\Delta\Delta Ct}$ method. *$P < .05$; **$P < .01$; ***$P < .005$; ****$P < .001$

was observed in an egg incubated for approximately 6–6.5 days.[19] Furthermore, the *sox9* gene, which is normally expressed in the developing vertebrate testis,[20] was observed to be expressed in the male gonad in an egg incubated for 6.0 days.[21] On the other hand, estrogen is required for ovary development in a female chicken embryo.[2] 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.[22,23] Male and female germ cells display different proliferative activity. During sex differentiation of the male gonad, there is virtually no germ-cell proliferation,[24] 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.[13] 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,[25] participates in the early sex-determination stage in chickens.[5] Moreover, another study identified a cell-autonomous sex-determination process in chickens.[6] 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′-GCCTCAACCCCTGCTTCCTC-3′ and 5′-CCGGC TCGGGCGGGCACTGG-3′, respectively. KOD FX (Toyobo Co. Ltd., Osaka, Japan) was used as a DNA polymerase for PC
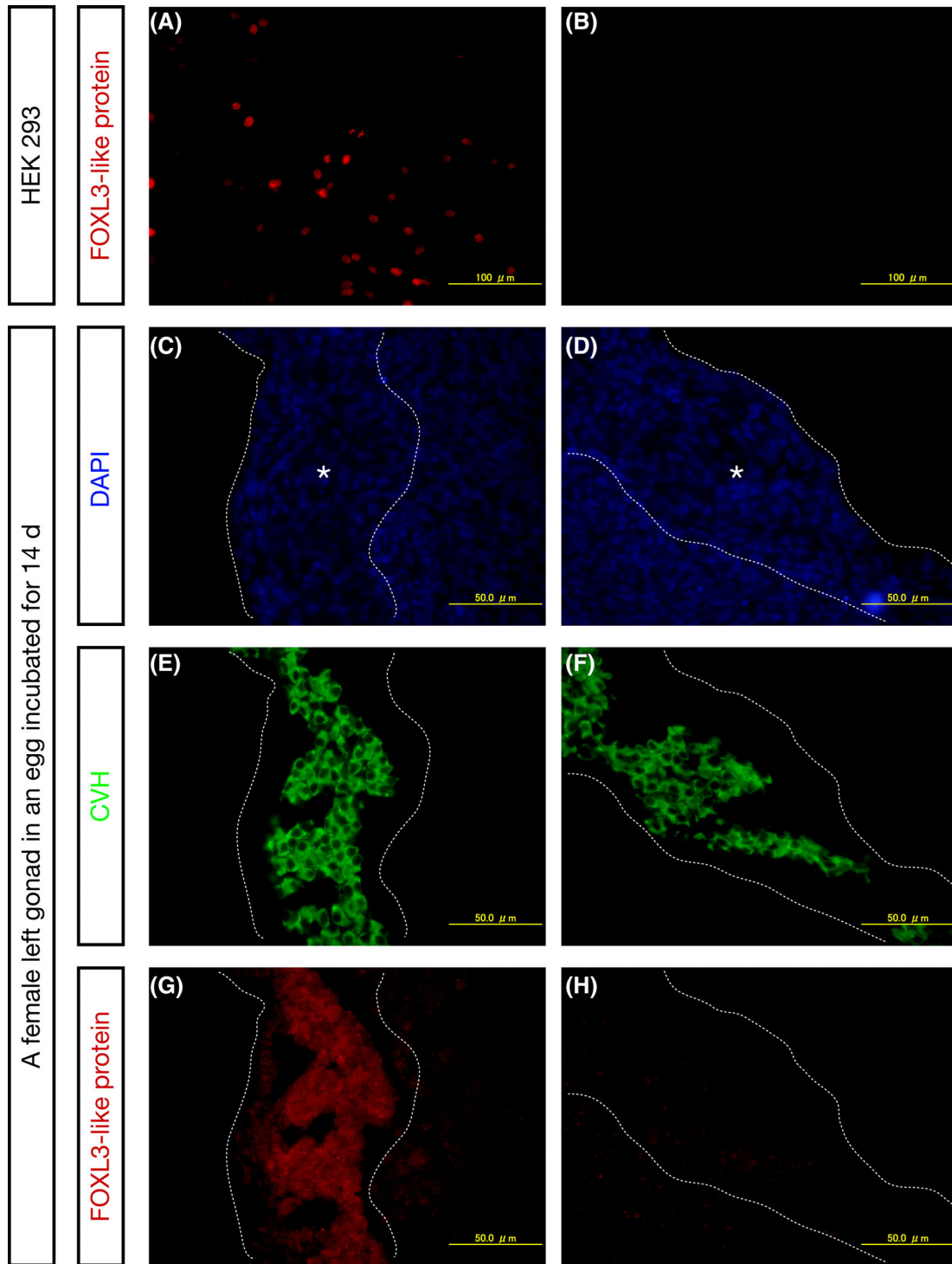
FIGURE 7 Immunofluorescence staining of the chicken FOXL3-like protein and CVH. (A, B) The specificity of the anti-chicken FOXL3-like protein antibody was evaluated by immunofluorescence staining in HEK293 cells expressing recombinant chicken FOXL3. (A) Staining with the anti-chicken FOXL3-like protein antibody or (B) total IgG purified from non-immunized serum. (C-H) Localization of the chicken FOXL3-like protein in the female left gonad in an egg incubated for 14 days and determined by immunofluorescence staining. Counterstaining of gonad sections with (C, D) DAPI, (E, F) the anti-CVH antibody, (G) the anti-chicken FOXL3-like protein antibody, or (H) total IgG purified from non-immunized serum. Cortex regions were divided by lines and marked with asterisks. Scale bars = 100 μm (A, B) and 50 μm (C-H)

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′-CGGCGGCGAATGGGC TCTGGAC-3′ and 5′-CGGGCTGCAGTGGGGGCGAAGC -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′-GGAGCGGCGGCGGAGGGAGATT-3′; and GeneRacer 5′, 5′-CGACTGGAGCACGAGGACACTGA-3′ was used. For the second amplification, primer c, 5′-GGACACTGAC ATGGACTGAAGGAGTA-3′ and 5′-GSP2, 5′-AGCGGC GGCGGCGGCAGTAATC-3′ were used. For the third amplification, primers c and d, 5′-GGAGGAAGCAGGGG TTGAGG-3′ (primer d) were used. The nucleotide sequences of 5′-GSP1, 5′-GSP2, and primer d were designed based on the sequence of the chicken *foxl3*-like gene cDNA fragment obtained from the F1-R1 primer PCR reaction. Step-down PCR was performed under the following conditions: five cycles of 98°C for 10 seconds and 74°C for 30 seconds, followed by five cycles of 98°C for 10 seconds and 72°C for 30 seconds, five cycles of 98°C for 10 seconds and 70°C for 30 seconds, and 20 cycles of 98°C for 10 seconds and 68°C for 30 seconds. KOD FX (Toyobo Co. Ltd.) was used as the DNA polymerase. PCR-product cloning and sequence analysis were performed as described.

The 3′ end of chicken *foxl3*-like gene was obtained by 3′ RACE using a GeneRacer Kit (Thermo Fisher Scientific). A poly(dT) adapter primer was used to reverse transcribe the mRNA [5′-GCTGTCAACGATACGCTACGTAACGGCA TGACAGTG(T)$_{18}$-3′] using PrimeScript reverse transcriptase (TaKaRa Bio). Three primer pairs were used to amplify the 3′ end of the chicken *foxl3*-like gene cDNA. For the first amplification, a primer pair (3′-GSP1 and GeneRacer 3′) was used. The sequence of the 3′-GSP1 primer was 5′-GGCTGCCGC TGCTCCGTGTTGC-3′, and GeneRacer 3′primer was 5′-GC

TGTCAACGATACGCTACGTAACG-3′. For the second amplification, 3′-GSP2, 5′-CACCTGGAGCCCGTCCTACTTC-3′, and GeneRacer 3'primers were used. For the third amplification, primers e, 5′-AGATCGGCTTCGCCCCCACTGC-3′ and f, 5′-CGCTACGTAACGGCATGACAGTG-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 $2^{-\Delta\Delta Ct}$ 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′-AATCCGGACCC TCCATTGTC-3′; and reverse, 5′-GCCATGCCAATCTCGTC 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 antibody[29] 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′-CCGGAATTCCGCGGGGCGATGGGGGACGAG-3′ and 5′-CCGCTCGAGGGGGCAGCACCGGGGCCCTGC-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 in TBST 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 total IgG purified from 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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