

## Oocytic expression of zona pellucida protein ZP4 in Japanese quail (*Coturnix japonica*)

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1 **Oocytic expression of zona pellucida protein ZP4 in Japanese quail (*Coturnix***  
2 ***japonica*)**

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12  
13 **Running title:** Cloning and expression of quail ZP4

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18

1 **Abstract**

2 The avian perivitelline layer, an extracellular matrix homologous to the zona pellucida  
3 of mammalian oocytes, is composed mainly by zona pellucida gene family glycoproteins.  
4 Our previous studies in Japanese quail have demonstrated that the matrix's components,  
5 ZP3 and ZPD, are synthesized in ovarian granulosa cells. Another component, ZP1, is  
6 synthesized in the liver. Recently, we demonstrated that another minor constituent, ZP2 is  
7 produced in the oocytes of the immature follicles. In the present study, we report the  
8 isolation of cDNA encoding quail ZP4 and its expression and origin in the female birds.  
9 By RNase protection assay and *in situ* hybridization, we demonstrated that ZP4 transcripts  
10 were transcribed in the oocytes of small white follicles. The expression level of ZP4  
11 decreased dramatically during follicular development, and the highest expression was  
12 observed in the small white follicles. Western blot analysis using the specific antibody  
13 against ZP4 indicated that the immunoreactive 58.2 kDa protein was present in the lysates  
14 of the small white follicles. These results demonstrate for the first time that the avian ZP4  
15 is expressed in the oocyte, and that the expression pattern of the gene is similar to that of  
16 ZP2.

17

18 *Keywords: egg envelope, Japanese quail, zona pellucida, ZP4,.*

19

## 1 INTORODUCTION

2 The egg envelope is an extracellular matrix that surrounds the oocyte of vertebrate  
3 animals and plays indispensable roles in gamete recognition during the initial process of  
4 fertilization, including species-specific sperm-egg binding, induction of the acrosome  
5 reaction and prevention of polyspermy (McLeskey *et al.* 1998). These egg envelopes are  
6 mainly constructed of glycoproteins belonging to different subclasses of the zona pellucida  
7 (ZP) gene family that possess a highly conserved amino acid sequence near the C terminus  
8 called the ZP domain (Bork & Sander 1992; Spargo & Hope 2003; Conner *et al.* 2005;  
9 Smith *et al.* 2005). The components of this matrix include three or four glycoproteins (*i.e.*,  
10 ZP1, ZP2, ZP3 and ZP4) in mammalian species (Litscher & Wassarman 2007). In rat  
11 (Hoodbhoy *et al.* 2005), human (Lefievre *et al.* 2004), bonnet monkey (Ganguly *et al.*  
12 2008) and hamster (Izquierdo-Rico *et al.* 2009) zona pellucida, 4 glycoproteins have been  
13 identified. However, in the case of mouse zona pellucida, only 3 glycoproteins have been  
14 identified because mouse ZP4 is a pseudogene due to the accumulation of stop codons  
15 (Conner *et al.* 2005). Amino acid sequence analysis reveals that ZP4 is most closely  
16 related to ZP1 because there is a trefoil domain in the upstream of the ZP domain (Conner  
17 *et al.* 2005). This indicate that the presence of ZP4 is not necessary for the formation of a  
18 functional zona pellucida, at least if ZP1 is present. In contrast to the mouse situation, it is  
19 very interesting to note that ZP1 has been identified as a pseudogene in the dog and cow  
20 genome (Goudet *et al.* 2008). In non-mammalian species, more than 4 ZP genes have been  
21 identified. For instance, there are 6 genes (ZP1, ZP2, ZP3, ZP4, ZPD and ZPAX) in the  
22 chicken genome (Goudet *et al.* 2008) and five genes (ZP2, ZP3, ZP4, ZPD and ZPAX) are  
23 found to be present in the genome of *Xenopus leivis* (Goudet *et al.* 2008).

24 At the time of ovulation, the avian oocyte is surrounded by the vitelline membrane, also

1 referred to as the perivitelline layer (PL), which is a rough meshwork of thick filaments  
2 mainly composed of glycoproteins (Wyburn *et al.* 1965). PL is a homologue of the egg  
3 envelope in other vertebrates, the zona pellucida in mammals. It has been reported that the  
4 avian PL is comprised of two major glycoproteins, ZP1 and ZP3, because after the  
5 separation of the PL constituents by SDS-PAGE, only two major bands (175 kDa and 35  
6 kDa in the quail and 183 kDa and 32 kDa in the chicken) identified as ZP1 and ZP3,  
7 respectively, were detected with Coomassie brilliant blue (CBB) staining (Mori & Masuda  
8 1993; Kido & Doi 1988). Previously, we identified 4 glycoproteins as the component of  
9 PL, ZP1, ZP2, ZP3 and ZPD in quail (Kinoshita *et al.* 2010; Pan *et al.* 2001; Sasanami *et al.*  
10 2003a; Sato *et al.* 2009) and that the ZP2 and the ZPD are present as a minor component in  
11 the PL. However, whether or not the ZP4 gene is actually expressed in the ovary is  
12 currently unknown, and if present, the question of which cells express this gene should be  
13 answered.

14 In the present study, we cloned quail ZP4 cDNA, and show that the ZP4 gene is  
15 expressed in the oocytes of the preovulatory follicles. Moreover, we also provide details  
16 about the expression pattern of the gene during the follicular development.

17

## 18 **MATERIALS AND METHODS**

### 19 **Animals and tissue preparation**

20 Female Japanese quail, *Coturnix japonica*, 15-30 weeks of age (Tokai-Yuki, Toyohashi,  
21 Japan), were maintained individually under a photoperiod of 14L: 10D (with the light on at  
22 05.00 hour) and were provided with water and a commercial diet (Tokai-Hokuriku Nosan,  
23 Chita, Japan) *ad libitum*. The animals were decapitated, and the preovulatory follicles  
24 were dissected and placed in physiological saline. All experimental procedures for the use

1 and the care of animals in the present study were approved by the Animal Care Committee  
2 of Shizuoka University (approval number, 21-23).

### 3 **cDNA cloning of quail ZP4**

4 The cDNA library of quail small white follicles (SWF, approximately 1 mm in  
5 diameter) was constructed with Creator SMART cDNA library construction kit according  
6 to the manufacturer's instructions (Clontech, Mountain View, CA, USA). Briefly, total  
7 RNA was extracted from the SWF with a commercial kit, RNAsiso (Takara Biomedicals,  
8 Otsu, Japan), according to the manufacturer's instructions, and mRNA was isolated using  
9 an oligotex-dT30 mRNA purification kit (Takara Biomedicals) as suggested by the  
10 manufacturer. The first strand cDNA was synthesized using a MMLV reverse  
11 transcriptase with CDS III primer in the presence of SMART IV oligonucleotide in order to  
12 introduce 5' and 3' primer sites containing *Sfi* I site into the 5' and the 3' end of the cDNA.  
13 Polymerase chain reaction (PCR) was performed using 5' and 3' primers, which supplied in  
14 the kit. PCR was performed in a 50  $\mu$  L volume with PCR buffer, 5 mmol/L MgCl<sub>2</sub>, 0.4  
15 mmol/L dNTP, and 2.5 U of Taq DNA polymerase. Following the addition of cDNA as the  
16 template, the reaction mixtures were incubated at 95 °C for 1 min and amplified for 22  
17 cycles. Each cycle consisted of 15 s denaturation at 95 °C and 6 min annealing and  
18 extension at 68 °C. The double strand cDNA in the amplicon was precipitated by ethanol,  
19 and was digested with *Sfi* I at 50 °C for 2 h. The digested DNA was separated with  
20 CHROMA SPIN-400 column, and the eluted fractions containing DNA were pooled and  
21 stored at -80°C as a cDNA insert. The cDNA insert was ligated into pDNR-LIB plasmid  
22 vector, and the product of the ligation reaction was introduced into an electro-competent  
23 cells (*DH5  $\alpha$* ; Takara Biomedical). They were then grown on the LB plate containing 30  
24  $\mu$  g/mL of chloramphenicol, and the antibiotics-resistant clone containing quail ZP4 was

1 selected after the nucleotide sequence analysis was performed on an Applied Biosystem  
2 Model 310 sequencer (Applied Biosystem, Foster City, CA, USA) by the dideoxy-mediated  
3 chain-termination method (Sanger *et al.* 1977).

#### 4 **Sequence computations**

5 Homology search by the position-specific iterated, basic local alignment search tool  
6 (PSI-BLAST) method (Altschul *et al.* 1997) and alignment of multiple protein sequences  
7 by the CLUSTAL W method (Thompson *et al.* 1994) were performed using the DNA Data  
8 Bank of Japan (<http://www.ddbj.nig.ac.jp>). The signal peptide and its putative cleavage site  
9 were predicted according to Nielsen *et al.* (1997) using SignalP v2  
10 (<http://www.cbs.dtu.dk/services/SignalP/>). The domain structures (ZP domain, trefoil  
11 domain and transmembrane domain) of the protein were predicted by pfam  
12 (<http://pfam.sanger.ac.uk/>) and SOSUI (<http://bp.nuap.nagoya-u.ac.jp/sosui/>) WWW server.

#### 13 ***RNase protection assay***

14 Total RNA was extracted from brain, heart, lung, liver, spleen, kidney and oviduct of  
15 laying female birds as described above. Total RNA was also obtained from the ovarian  
16 tissue containing very small white follicles (VSWF, less than 1 mm in diameter), SWF,  
17 large white follicles (LWF, 2-3 mm in diameter) or small yellow follicles (SYF, 3-10 mm  
18 in diameter). In the case of the large yellow follicles (LYF, more than 10 mm in diameter)  
19 the follicular walls were peeled off by fine forceps, and were processed for RNA extraction.

20 PCR-amplified fragments derived from quail ZP4 cDNA (314 nt; 344-657 of GenBank  
21 accession number AB458445) or quail S17 ribosomal protein cDNA (Yamamoto *et al.*  
22 2003) were subcloned into a pGEM-3Z vector (Promega Corporation, Madison, WI, USA).  
23 After linearization with the appropriate restriction enzymes, radioactive antisense or sense  
24 riboprobes were synthesized using Riboprobe in vitro Transcription System (Promega

1 Corporation) and [ $\alpha$ -<sup>32</sup>P] CTP (NEN Life Science Products, Boston, MA, USA). The RNA  
2 probes were hybridized with total RNA overnight and digested with RNase A and T1  
3 (Tanaka *et al.* 1999). The protected RNAs were separated on 6% (w/v)  
4 polyacrylamide/urea gels. The radioactive bands on the dried gels were detected with  
5 autoradiography with the aid of an intensifying screen (Kodak, Rochester, NY, USA).

### 6 ***In situ* hybridization**

7 The birds were decapitated, the follicles were immediately removed, and frozen  
8 sections were prepared. *In situ* hybridization was carried out as described previously  
9 (Yoshimura *et al.* 2000). The antisense 45 mer oligonucleotide probe (nucleotides 811-856  
10 of GenBank accession number AB458445) was labeled with [<sup>33</sup>P] dATP (NEN Life  
11 Science Products) using terminal deoxyribonucleotidyl transferase (Gibco, Frederic, MD,  
12 USA). The sense probe was also labeled as described above. Hybridization was carried  
13 out overnight at 42°C. Washing was performed at room temperature for 30 min and at  
14 55°C for 40 min twice. After washing, slides were exposed to Biomax-MR film (Kodak,  
15 Rochester, NY, USA) for 2 weeks. After the exposure, the slides were coated with Kodak  
16 NTB-2 emulsion (Kodak), and exposed for 7 days at 4°C in a dark box. After exposure,  
17 the slides were developed in Kodak Dektol and mounted in glycerol. The specimens were  
18 examined under a dark-field microscopy with an X 40 objective (Nikon, Tokyo, Japan).

### 19 **Production of antiserum against quail ZP4**

20 Rabbit polyclonal anti-quail ZP4 antibody was raised against bacterially expressed  
21 His-tag fused with partial ZP4 protein (from Ser<sup>32</sup> to Ala<sup>196</sup>). Quail ZP4 cDNA was  
22 amplified by PCR (cycling conditions: 94 °C for 30 sec, 58.3 °C for 30 s, and 72 °C for 45  
23 s for 35 cycles) in order to introduce *Eco*R I and *Xba* I sites upstream of the Ser<sup>32</sup> and  
24 downstream of the Ala<sup>196</sup>, respectively. The sense and antisense primers used were 5'-

1 AAAAGAATTCAGCCCTTTGGCTGACCCTGGC-3' and 5'-  
2 AAAATCTAGATGCTGTCACCTGTGTTACCATA-3', respectively. The PCR product  
3 containing the partial quail *ZP4* cDNA was digested with *EcoR* I and *Xba* I, and ligated  
4 into pCold TF DNA vector (Takara Biomedical) treated with the same restriction enzymes.  
5 The resulting construct was transformed into competent *Escherichia coli*, strain *BL21*  
6 (Takara Biomedical), and an ampicillin-resistant clone was selected after the nucleotide  
7 sequence analysis was performed. Recombinant quail *ZP4* was expressed as the 69.8 kDa  
8 fusion protein (17.8 kDa of the partial *ZP4* protein with 52 kDa of the trigger factor plus  
9 the His tags) in the presence of 1 mmol/L Isopropyl- $\beta$ -thiogalactopyranoside at 15°C for 24  
10 h, and the protein was purified from the cell lysate using nickel resin (Novagen, Madison,  
11 WI, USA) according to the manufacturer's instructions. The purity of the recombinant *ZP4*  
12 was verified by separating the protein by SDS-PAGE followed by CBB staining.

13 A single female New Zealand White rabbit (SLC, Hamamatsu, Japan) was immunized  
14 with the recombinant quail *ZP4* (300  $\mu$ g of protein) as described previously (Kuroki &  
15 Mori 1995).

#### 16 **Gel electrophoresis and Western blot analysis**

17 The VSWF, SWF, LWF or SYF was homogenized in the ice-cold extraction buffer  
18 containing 2 mmol/L CaCl<sub>2</sub>, 150 mmol/L NaCl and 1 mmol/L PMSF buffered at pH 6.0  
19 with 20 mmol/L Tris-HCl, and a debris was precipitated by centrifugation at 5,000 x *g* for 5  
20 min at 4°C. The supernatants were further centrifuged at 100,000 x *g* for 1h at 4°C in order  
21 to remove the insoluble materials. The clear supernatants were served as follicular  
22 extracts and stored at -80°C until used. The protein concentration in each sample was  
23 determined using a BCA Protein Assay kit (Pierce, Rockford, IL, USA).

24 SDS-PAGE under reducing conditions was carried out as described previously

1 (Laemmli 1970), using 12 and 5% (w/v) polyacrylamide for resolving and stacking gels,  
2 respectively. For Western blotting, proteins separated on SDS-PAGE were transferred to a  
3 polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA,  
4 USA) (Matsudaira 1987). The membrane reacted with anti-quail ZP4 antiserum (1:1, 000)  
5 was visualized by means of a chemiluminescent technique (Amersham Pharmacia Biotech,  
6 Piscataway, NJ, USA) using horseradish peroxidase-conjugated anti-rabbit IgG (Cappel,  
7 Durham, NC, USA) as a secondary antibody.

8

## 9 **RESULTS**

### 10 **Cloning of quail ZP4**

11 In order to find the putative ZP4 gene in our cDNA library, we performed DNA  
12 sequencing of the 120 cDNA clones. We focused on the cDNA clone, which possesses 1.5-  
13 2.0 kbp in their size because the size of the chicken ZP4 had been reported to be 1678 bp  
14 (GenBank accession number AB025428). As the result, we obtained the cDNA clone,  
15 which showed a high degree of similarity in the DNA sequence to chicken ZP4 cDNA.  
16 The sequences have been deposited in the GenBank database (accession number:  
17 AB458445). As shown in Figure 1A, the DNA sequence of ZP4 is 1741 nucleotides long  
18 and contains a single open reading frame. This sequence contains an ATG initiation codon  
19 and a stop codon (TGA) in positions at 1-3 and 1633-1635, respectively and a  
20 polyadenylation signal (AATAAA) is found in positions at 1664-1669.

21 The isolated cDNA encoded 544 amino acids with a theoretical molecular weight of  
22 58.956 kDa, including a predicted 31-amino acid signal peptide (Fig. 1A). As with other  
23 ZP proteins (ZP1, ZP2, ZP3 or ZPD), ZP4 contains a ZP domain (His<sup>197</sup>-Arg<sup>471</sup>) and a  
24 consensus furin-cleavage site (Arg<sup>473</sup>-Gly<sup>474</sup>-Arg<sup>475</sup>-Arg<sup>476</sup>) immediately after the ZP

1 domain, indicating the possibility that the C-terminal region of the newly synthesized  
2 precursor protein could be proteolytically processed by furin-like protease. In addition to  
3 the ZP domain, the prediction of the domain structure reveals that the quail ZP4 contains a  
4 trefoil domain in the upstream of the ZP domain (Val<sup>150</sup>-Gly<sup>191</sup>). The molecular mass of  
5 the processed peptide (with no signal peptide and cleavage at Arg<sup>476</sup> in the C-terminal  
6 region of ZP4 protein) was calculated to be 48.502 kDa. The sequences showed a high  
7 degree of hydrophobicity at the C-terminal region corresponding to the transmembrane  
8 domain between Trp<sup>518</sup> and Leu<sup>540</sup>, which is followed by a short cytoplasmic tail.  
9 Interestingly, unlike other ZP proteins, quail ZP4 possesses an additional transmembrane  
10 domain in the upstream of the ZP domain (Val<sup>95</sup>-Leu<sup>117</sup>). Because we could not find an  
11 additional protease-sensitive sequence near the N-terminal transmembrane domain, it  
12 indicates the possibility that the quail ZP4 might show type II membrane topology in the  
13 plasma membrane of the cells.

14 Quail ZP4 was compared with ZP4 protein from domestic fowl or rat (Fig. 1B). The  
15 quail ZP4 is closely related to its chicken counterpart. The amino acid sequence show a  
16 high degree of homology to the chicken ZP4 protein (89.0 %), but it is partially related to  
17 rat counter part (45.9 %). There are 2 putative *N*-glycosylation sites in the translated  
18 protein that are completely conserved with chicken homologue. 10 conserved Cys residues  
19 were seen in the ZP domain of the ZP4 of all three species, and 6 Cys are also common in  
20 the trefoil domains of chicken, quail and rat ZP4, suggesting that their 3-dimensional  
21 structures are similar to each other. These common features suggest that our clone is an  
22 authentic member of ZP4.

### 23 **mRNA expression of ZP4 in quail**

24 To determine the tissue distribution of *ZP4* transcripts, we analyzed the total RNA

1 isolated from various tissues of laying quail using a gene-specific RNase protection assay  
2 (Fig. 2). As shown in the figure, an intense single protected fragment was detected in the  
3 VSWF of the ovary (lane 12). It should be noticed that the size of the protected fragment  
4 was smaller than that of the probe itself (lane 13) because the RNase treatment can remove  
5 the unhybridized regions, which are derived from the pGEM-3-z vector during the  
6 synthesis of RNA probes. A faint band was seen in the sample prepared from SWF, LWF,  
7 SYF and LYF at the same position as that of the VSWF, and no such signal above the  
8 background was observed when the sample was obtained from any other tissues (lanes 1-7).  
9 Equal loading of RNA onto gels was verified by hybridization of the sample with the probe  
10 for S17 ribosomal protein (data not shown). These results demonstrated that the  
11 expression of ZP4 gene is restricted to the ovary of Japanese quail.

12 Next, we performed the *in situ* hybridization in order to confirm the temporal expression  
13 pattern of ZP4 transcripts in the ovary during follicular development. In accord with the  
14 data in the RNase protection assay, the intense signal was detected in the VSWF and SWF  
15 (less than 1 mm in diameter, Fig.3A, arrowheads). The peripheral region of LWF was also  
16 faintly labeled with our probe (Fig.3A, arrows). No such signals were seen in the case of  
17 the developing follicles including SYF or LYF. When the specimens were hybridized with  
18 sense probe, again, no such intense signal was detected (Fig. 3B), confirming the specificity  
19 of the hybridization. These results clearly suggest that the expression of the ZP4 gene in  
20 the follicles is dramatically decreased during follicular development.

21 To further localize the expression of ZP4 transcripts in the follicles, we observed the  
22 hybridized-specimens under dark-field microscopy (Fig. 3C). As shown in the figure, the  
23 intense signals were observed in the oocyte of the VSWF, suggesting that the ZP4 mRNA  
24 appears to be transcribed in the oocyte itself. The specimens that had been hybridized with

1 the sense probe did not contain these radio-labeled signals (data not shown). The bright  
2 field observations revealed that the ZP4 mRNA in the LWF is also located in the oocyte,  
3 though the intensity of the signal is far weaker than that of VSWF (Fig. 3D). These results  
4 demonstrate for the first time that the expression of the ZP4 gene is restricted in the oocytes  
5 and that the expression is decreased during follicular development.

### 6 **Detection of ZP4 protein**

7 To confirm the presence of the ZP4 protein in the follicle, we produced an antiserum  
8 against bacterially expressed quail ZP4. We immunized the purified recombinant ZP4 to a  
9 rabbit, and the antiserum against quail ZP4 was obtained. To visualize the ZP4 protein in  
10 the follicular extracts, we performed Western blot analysis. As shown in Figure 4, our anti-  
11 ZP4 antibody reacted well with the band with 58.2 kDa in the VSWF and SWF extract  
12 under reducing conditions (panel A, lanes 1 and 2). This size is far greater than that of the  
13 theoretical molecular weight of mature ZP4 protein (48.502 kDa). It is probably due to the  
14 addition of oligosaccharides moieties during the post-translational modification of the ZP4  
15 protein in the cells. No band was detected when the sample was reacted with preimmuno  
16 serum (panel B), which suggests that our anti-ZP4 antibody is specific to the 58.2 kDa  
17 proteins. These results suggest that the immunoreactive materials that were recognized  
18 with anti-ZP4 antibody exist in the extract of the follicles.

19

### 20 **DISCUSSION**

21 In a previous study, we found that quail granulosa cells produce ZP3 and ZPD, which  
22 are the components of the PL (Pan *et al.* 2001; Sato *et al.* 2009), whereas another major  
23 constituent, ZP1, is synthesized in the liver (Sasanami *et al.*, 2003a). A recent study  
24 revealed that another minor constituent, ZP2, is produced in the oocytes themselves in

1 Japanese quail (Kinoshita *et al.* 2010). In the present study, we clearly demonstrated for  
2 the first time that the mRNA for ZP4 is expressed in the oocytes of immature follicles in  
3 the quail ovary.

4 From the deduced amino acid sequence analysis of quail ZP4, we found that there is a  
5 transmembrane domain in the N-terminal region upstream of its ZP domain in addition to  
6 the C-terminal one. The C-terminal transmembrane domain, which is common in all  
7 known ZP glycoproteins except for fish ZP glycoproteins (Rankin & Dean 2000; Jovine *et*  
8 *al.* 2005), is suggested to be involved in the modification of precursor to form the mature  
9 ZP glycoprotein in various species (Litscher *et al.* 1999; Kubo *et al.* 1999; Sasanami *et al.*  
10 2002; Yonezawa & Nakano 2003). In addition to the role for the biosynthesis of ZP  
11 glycoproteins, it is also reported that the removal of the C-terminal transmembrane domain  
12 controls the secretion/release of the ZP glycoprotein from the cells in mice (Jovine *et al.*  
13 2002, 2004, 2005). The results of our previous study in Japanese quail also suggest this C-  
14 terminal proteolytic processing and removal of transmembrane domain is a prerequisite for  
15 ZP3 secretion from the cells (Sasanami *et al.* 2003b). Although the participation of the  
16 transmembrane domain for ZP4 biosynthesis has not yet been elucidated, it is quite  
17 interesting to investigate the role of N-terminal transmembrane domain in the ZP4  
18 production, since the presence of two transmembrane domains in the sequence is the unique  
19 feature of avian ZP4 protein.

20 In spite of the structural and functional similarities of ZP glycoproteins, the sources of  
21 ZP glycoproteins are different in different species (Menkhorst & Selwood 2008). In most  
22 mammalian and amphibian species, it is clearly demonstrated that ZP glycoproteins are  
23 synthesized by the growing oocytes (Bleil & Wassarman 1980; Yamaguchi *et al.* 1989). In  
24 fish, however, egg envelope glycoproteins homologous to ZP glycoproteins are produced in

1 the liver and transported to the ovary by the blood circulation, like vitellogenin (Hamazaki  
2 *et al.* 1985, 1989). Our present and previous experimental evidences strongly suggest that  
3 female germ cells (*i.e.*, the oocyte) also participate in producing the ZP glycoprotein in  
4 addition to the ovarian granulosa cells (ZP3 and ZPD) and the liver (ZP1) in avian species.  
5 We have no rational explanation why three types of cells should participate in the  
6 formation of the egg coat in avian species, however, it might be because avian oocytes  
7 grow very rapidly during final stage of the follicular development, and that an active organ  
8 in protein synthesis, such as liver, should participate in the synthesis of the ZP  
9 glycoproteins together with ovarian somatic and germ cells in order to overlie the surface  
10 of the big oocytes in avian species. This speculation is supported by the observation that  
11 the accumulation of ZP1 protein occurs at later stage of the follicular development when  
12 the thickness of the PL increases dramatically (Sasanami *et al.* 2004). The ZP1 proteins  
13 might render the mechanical strength to the egg coat at the time of ovulation, in addition to  
14 its indispensable roles for the induction of the sperm acrosome reaction during the  
15 fertilization (Sasanami *et al.* 2007).

16 In the present study, we found that the transcripts of ZP4 protein in the follicles  
17 decreased during follicular development (Fig. 2C). This pattern is very different from that  
18 for the ZP glycoproteins derived from somatic cells (*i. e.*, the granulosa cells and the  
19 hepatocytes), in that the ZP3 accumulates in the PL obtained from the SYF, and the ZP1  
20 and ZPD accumulate thereafter (Pan *et al.* 2001; Sato *et al.* 2009; Sasanami *et al.* 2004).  
21 On the other hand, the expression pattern of the ZP4 is quite analogous to that of ZP2  
22 expressed in the germ cells in which the highest expression is observed in the immature  
23 follicles, VSWF and SWF, and is dramatically decreased thereafter (Kinoshita *et al.* 2010).  
24 Two sets of the ZP glycoproteins whose expression are reciprocally controlled during

1 oocyte development rendered us to hypothesize that the ZP2 and ZP4 proteins might  
2 function in the immature oocyte, whereas, another ZP glycoproteins (ZP1, ZP3 and ZPD)  
3 might play a role in the mature one for the formation of the PL. In fact, in vitro incubation  
4 of ZP1 with ZP3 spontaneously produced fibrous aggregates, which were visible under  
5 optical microscopy (Okumura *et al.* 2007). We also observed that the intravenously  
6 injected-ZP1 is more actively incorporated into the PL of the mature follicles than that of  
7 the immature ones (Kinoshita *et al.* 2008). In addition, in our recent study, we observed a  
8 detectable interaction of ZP2 and ZP3 by means of Western blot analysis and  
9 immunofluorescence microscopy suggesting that the ZP2 protein localized on the surface  
10 of the immature oocyte might be one of a target molecule for ZP3 binding, and that this  
11 interaction might trigger the ZP glycoprotein accumulation (Kinoshita *et al.* 2010).  
12 Although the direct experimental evidence suggesting the role of ZP4 in the PL formation  
13 is not available, we expect that the ZP4 protein might interact with one of the ZP  
14 glycoproteins and this interaction might trigger the PL formation together with ZP2 protein.  
15 Additional investigations are needed to understand the mechanism of the formation of the  
16 PL fibers during follicular maturation.

17

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22

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1 **Figure captions**

2 Figure. 1 (A) Nucleotide and deduced amino-acid sequences of quail ZP4 cDNA.  
3 The deduced amino acid is represented as a single-letter abbreviation shown below the  
4 nucleotide sequence (GenBank Accession Number: AB458445). The signal sequence  
5 (italic letters), the zona pellucida domain (ZP domain, white box), the trefoil domain  
6 (shaded box), the consensus site for *N*-glycosylation (bold letters), the consensus furin  
7 cleavage site (bold letters with underlining), a poly-adenylation signal sequence  
8 (underlining), and the putative transmembrane domain (black box) are indicated. The  
9 asterisk indicates the termination codon. Primer positions used for amplifying the probe for  
10 RNase protection assay (double underlines) and the antigen production for anti-ZP4  
11 antiserum (hatched underlines) and probe for in situ hybridization (bold underline) are  
12 shown. (B) Alignment of amino acid sequence of quail, chicken and rat ZP4. Conserved  
13 residues are shown in a shaded box. The consensus site for *N*-glycosylation (double  
14 underlining), conserved Cys residues (bold letters) and the consensus furin cleavage site  
15 (underlining) are indicated.

16 Figure. 2 Tissue-specific expression of ZP4 mRNA. RNase protection assays were  
17 performed to detect the mRNA expression of the ZP4 gene. We hybridized 15 µg of total  
18 RNA isolated from brain (lane 1), heart (lane 2), lung (lane 3), liver (lane 4), spleen (lane 5),  
19 kidney (lane 6), oviduct (lane 7), LYF (lane 8), SYF (lane 9), LWF (lane 10), SWF (lane  
20 11) and VSWF (lane 12) with riboprobes specific to ZP4. As a reference, the antisense  
21 probe used in this experiment was run on the right lane (lane 13). Protected fragments were  
22 detected by autoradiography. The results shown are representative of repeated experiments.  
23 M: size marker.

24 Figure. 3 *In situ* hybridization of quail ovary. (A) Autoradiogram of ovarian sections after

1 hybridization with <sup>33</sup>P-labeled antisense probe specific to ZP4 is shown. Arrowheads and  
2 arrows indicate the VSWF/SWF and the LWF, respectively. (B) Control autoradiogram  
3 hybridized with <sup>33</sup>P-labeled sense probe is depicted. Scale bar, 5 mm. (C) The slides  
4 hybridized with antisense probe were coated with Kodak NTB-2 emulsion, and examined  
5 under a dark-field microscopy. The follicles having the silver grains in the oocytes of  
6 VSWF are visualized. Scale bar, 100 μm. The results are representative of repeated  
7 experiments. (D) Similarly hybridized specimen in bright field. The slides shown in panel  
8 C were observed in bright field. Note that the silver grains of the left follicle (SWF)  
9 localized in the apical region of the oocyte beneath the granulosa layers. Th; theca layer,  
10 G; granulosa layer, O; oocyte. Scale bar, 50 μm. The results are representative of repeated  
11 experiments.

12 Figure. 4 Western blot analysis of ZP4 protein in the extract of the follicles. The  
13 extracts prepared from the SYF (lane 1), the LWF (lane 2), the SWF (lane 3) or the VSWF  
14 (lane 4) (5 μg /lane) were separated on SDS-PAGE under reducing conditions, transblotted  
15 onto PVDF membrane, and detected with anti-quail ZP4 antiserum (panel A, 1: 1, 000) or  
16 preimmunoserum (panel B, 1: 1, 000). The results are representative of repeated  
17 experiments.