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doi: 10.1111/j.1740-0929.2010.00830.x

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

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Running title: Cloning and expression of quail ZP4

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Abstract

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

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

INTORODUCTION

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

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

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

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

MATERIALS AND METHODS

Animals and tissue preparation

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

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

cDNA cloning of quail ZP4

The cDNA library of quail small white follicles (SWF, approximately 1 mm in diameter) was constructed with Creator SMART cDNA library construction kit according to the manufacturer's instructions (Clontech, Mountain View, CA, USA). Briefly, total RNA was extracted from the SWF with a commercial kit, RNAiso (Takara Biomedicals, Otsu, Japan), according to the manufacturer's instructions, and mRNA was isolated using an oligotex-dT30 mRNA purification kit (Takara Biomedicals) as suggested by the manufacturer. The first strand cDNA was synthesized using a MMLV reverse transcriptase with CDS III primer in the presence of SMART IV oligonucleotide in order to introduce 5' and 3' primer sites containing *Sfi* I site into the 5' and the 3' end of the cDNA. Polymerase chain reaction (PCR) was performed using 5' and 3' primers, which supplied in the kit. PCR was performed in a 50 μL volume with PCR buffer, 5 mmol/L $MgCl_2$, 0.4 mmol/L dNTP, and 2.5 U of Taq DNA polymerase. Following the addition of cDNA as the template, the reaction mixtures were incubated at 95℃ for 1 min and amplified for 22 cycles. Each cycle consisted of 15 s denaturation at 95℃ and 6 min annealing and extension at 68℃. The double strand cDNA in the amplicon was precipitated by ethanol, and was digested with *Sfi* I at 50℃ for 2 h. The digested DNA was separated with CHROMA SPIN-400 column, and the eluted fractions containing DNA were pooled and stored at -80℃ as a cDNA insert. The cDNA insert was ligated into pDNR-LIB plasmid vector, and the product of the ligation reaction was introduced into an electro-competent cells (*DH5* α; Takara Biomedical). They were then grown on the LB plate containing 30 μg/mL of chloramphenicol, and the antibiotics-resistant clone containing quail ZP4 was

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

Sequence computations

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

RNase protection assay

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

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

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

In situ hybridization

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

Production of antiserum against quail ZP4

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

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

A single female New Zealand White rabbit (SLC, Hamamatsu, Japan) was immunized with the recombinant quail ZP4 (300 μg of protein) as described previously (Kuroki & Mori 1995).

Gel electrophoresis and Western blot analysis

The VSWF, SWF, LWF or SYF was homogenized in the ice-cold extraction buffer containing 2 mmol/L $CaCl_2$, 150 mmol/L NaCl and 1 mmol/L PMSF buffered at pH 6.0 with 20 mmol/L Tris-HCl, and a debris was precipitated by centrifugation at 5,000 x *g* for 5 min at 4℃. The supernatants were further centrifuged at 100,000 x *g* for 1h at 4℃ in order to remove the insoluble materials. The clear supernatants were served as follicular extracts and stored at -80℃ until used. The protein concentration in each sample was determined using a BCA Protein Assay kit (Pierce, Rockford, IL, USA).

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

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

RESULTS

Cloning of quail ZP4

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

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

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

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

mRNA expression of ZP4 in quail

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

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

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

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

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

Detection of ZP4 protein

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

DISCUSSION

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

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

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

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

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

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

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

Acknowledgments

This work was supported in part by a Grant-in-Aids for Scientific Research (21658100 to T Sasanami) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Figure captions

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

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

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

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

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