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Assembly of the Inner Perivitelline Layer, a Homolog of the Mammalian Zona Pellucida: An Immunohistochemical and Ultrastructural Study

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Key Words
Oocyte · Inner perivitelline layer · Immunohistochemistry · Ultrastructure · Quail

Abstract
The avian inner perivitelline layer (IPVL), a homologous structure to the mammalian zona pellucida, is deposited between the granulosa cells and the oocyte cell membrane during folliculogenesis. The glycoprotein meshwork of the IPVL forms a 3-dimensional matrix and possesses important functions in the fertilization process: it contributes to the binding of avian spermatozoa to the oocyte and induces acrosomal exocytosis. In contrast to the zona pellucida of mammals, the IPVL does not prevent the physiological polyspermy found in birds. Previous studies have shown that in the Japanese quail *(Coturnix japonica)* at least 5 glycoproteins are constituents of the IPVL (ZP1, ZP2, ZP3, ZP4, and ZPD). In this study, we investigated the spatiotemporal assembly pattern of the IPVL during folliculogenesis using immunohistochemical and ultrastructural methods. The obtained results clearly show that these glycoproteins are incorporated into the IPVL at distinct points during follicular development, supporting the hypothesis that ZP2 and ZP4 form a type of prematrix into which ZP1, ZP3, and ZPD are integrated at a later stage of development.

Introduction

The oocyte cell membrane of all vertebrates is surrounded by a filament network mainly composed of glycoproteins that form a 3-dimensional extracellular matrix [Wyburn et al., 1965a, b]. This matrix is deposited between the maturing oocyte and the granulosa cells during follicular growth. Different names have been proposed for this acellular layer, depending on the class of animals: 'zona pellucida' (ZP) in mammals, 'chorion' in teleosts, 'vitelline membrane' in amphibians and chondrichthyans, and, depending on the author, '(inner) perivitelline layer'

Abbreviations used in this paper

F1 (2, 3, 4, 5)	follicular stage 1 (2, 3, 4, 5)
IPVL	inner perivitelline layer
OPVL	outer perivitelline layer
PBS	phosphate buffered saline
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SWF	small white follicle
SYF	small yellow follicle
ZP	zona pellucida
ZP1 (2, 3, 4, D)	zona pellucida protein 1 (2, 3, 4, D)

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(IPVL), 'perivitelline membrane', or 'vitelline membrane' in reptiles and birds. Although distinct morphological differences exist between the different vertebrate classes, these layers share considerable biochemical and functional similarities [Wyburn et al., 1965a; King, 1975; Spargo and Hope, 2003; Stewart et al., 2004; Menkhorst and Selwood, 2008]. These similarities exist because these layers contain glycoproteins, all encoded by different members of the ZP gene family, that possess highly conserved amino acid sequences and are structurally closely related [Spargo and Hope, 2003; Jovine et al., 2005; Smith et al., 2005].

In mammals, the thickness of this extracellular matrix varies from 1 to 2 μm in the opossum, from 7 to 11 μm in horses and pigs, and from 13 to 16 μm in humans, and it can measure up to 27 μm in bovines [Dunbar et al., 1994; Mugnier et al., 2009]. The IPVL of birds possesses an average thickness of 1–3 μm and is located between a monolayer of granulosa cells and the ooplasma membrane of growing oocytes, amidst the zona radiata. The latter is composed of cytoplasmic processes of granulosa cells and the oocyte, similar to mammals, recedes before ovulation [Wyburn et al., 1965a, b].

Despite characteristic morphological features, egg covers of different animal classes (such as birds and mammals) show similar functions, including species-specific sperm-egg binding and induction of the acrosomal reaction [Wyburn et al., 1965a; King, 1975; Spargo and Hope, 2003; Stewart et al., 2004; Menkhorst and Selwood, 2008]. However, distinct differences are also observed. For instance, the polyspermy observed in avian species is physiologic and does not interrupt the development of the embryo [King, 1975]. In birds, the early embryo also does not hatch from the zona equivalent, the IPVL. In addition to its important roles during the fertilization process, the avian IPVL (also termed the 'vitelline membrane inner layer' by Burley and Vadehra [1989]) serves as a supporting layer for the adjacent outer perivitelline layer ('vitelline membrane outer layer'). The latter provides the innermost part of the chalaziferous layer of the bird's egg. During its passage through the oviduct, this subpart of the chalaziferous layer, together with the IPVL, participates in anchoring the chalazae which adjust the eggshell rotation for the embryonic body axis determination [Rahman et al., 2007, 2009].

The mammalian ZP is known to usually be composed of 4 glycoproteins, i.e. ZP1, ZP2, ZP3, and ZP4 [Spargo and Hope, 2003; Smith et al., 2005]. These glycoproteins may be exclusively formed by the oocyte, as in the mouse [Epifano et al., 1995], or by the cooperation of oocyte and granulosa cells, as in various domestic animal species.

Five glycoproteins have so far been detected to be regular constituents of the IPVL in the Japanese quail *(Coturnix japonica),* with ZP1 and ZP3 being the major components and ZP2, ZP4, and ZPD being the minor components (fig. 1) [Mori and Masuda, 1993; Waclawek et al., 1998; Takeuchi et al., 1999; Pan et al., 2001; Sasanami et al., 2003; Sato et al., 2009; Kinoshita et al., 2010; Serizawa et al., 2010]. These glycoproteins, which are synthesized in 3 different locations (the oocyte, granulosa cells, and liver) are incorporated into the forming IPVL to establish a dense, protective, and resistant extracellular matrix [Sasanami et al., 2004; Litscher and Wassarman, 2007]. In the present study, the precise time course of the IVPL assembly in the quail was studied using immunohistochemical and ultrastructural techniques.

Materials and Methods

Animals and Tissue Preparation

Twenty-five healthy, female, 6-month-old, regularly laying Japanese quails *(C. japonica)* were used in this study. The birds were provided by the Institute of Veterinary Nutrition, Department of Veterinary Sciences, University of Munich, Germany. All birds were housed as indoor breeders with an illumination continuity of 16 h per day. They were fed with a commercial diet and water ad libitum. Tissue was collected 8 h before ovulation. Because no experimental procedures were involved in this study, the German Law on Animal Welfare (§§8b, 9.2) and the Animal Care Committee of the Veterinary Faculty of the University of Munich authorized the sacrifice of the quails for scientific use. Under CO_2 anesthesia, the quails were decapitated and their ovaries removed and fixed in Bouin's solution (1,500 ml picric acid, 500 ml glacial acetic acid, and 100 ml 37% formalin) for 14 h. Then, tissue samples of different follicular sizes of the ovary were cut and dehydrated in a graded series of ethanol and embedded using an automatic tissue processor (Shandon Duplex Processor; Frankfurt, Germany) and a Histo Stat Tissue Embedding Center (Reichert-Jung, Vienna, Austria). Paraplast sections of a thickness of 5 μm were cut using a rotary microtome (Leitz Microm HM 340E; Wetzlar, Germany). Serial sections were collected on silane-coated glass slides (Menzel-Glaeser, Braunschweig, Germany).

Immunohistochemistry

Paraffin sections were dewaxed in xylene, rehydrated, and washed with PBS buffer (pH 7.4). All following incubation steps were performed in a humidified chamber. Endogenous peroxidase activity was blocked with 0.1% H_2O_2 for 10 min. After washing with PBS buffer, the sections were incubated with antibodies against the following polyclonal antigens: ZP1, ZP2, ZP3, ZP4, and ZPD [Sasanami et al., 2002, 2004; Sato et al., 2009; Kinoshita et al., 2010; Serizawa et al., 2010] (University of Shizuoka, Japan) for 16 h at 4°C, followed by incubation with a secondary antibody (anti-rabbit, biotinylated, species pig; Dako, Hamburg, Germany) for 30 min at 20°C.

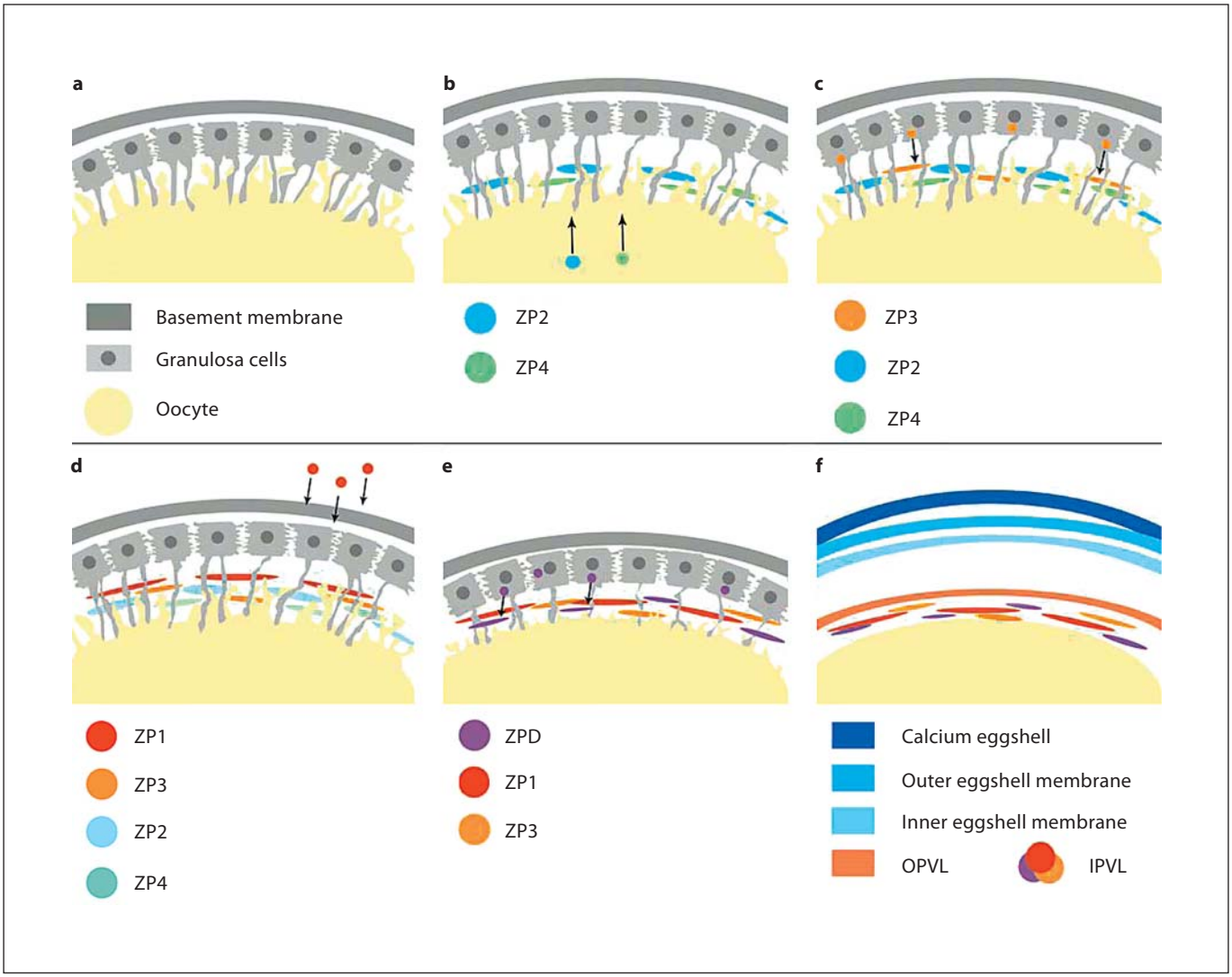

Fig. 1. Immunohistochemical model of IPVL development in the Japanese quail. **a** Primordial follicle. In primordial and early previtelline follicles, the granulosa cells and the oocyte are interconnected by long cytoplasmic processes and microvilli. No IPVL has developed at this stage. **b** Later previtelline follicles and SWF (≤1 mm). ZP2 and ZP4, synthesized by the oocyte, are incorporated into the extracellular space between the apical surface of the granulosa cells and the ooplasma membrane of the oocyte. **c** Follicles at the SYF stage (1–2 mm). ZP3, a major component of the IPVL and synthesized by the granulosa cells, is integrated into the ZP2-ZP4 prematrix at this follicular stage. **d** Vitelline follicles at the F5 stage (2–4 mm). The liver synthesizes ZP1, which is transported via the blood stream and incorporated into the IPVL at this follicular stage as a comparatively large matrix component. The immunohistochemical signals of ZP2 and ZP4 become weaker at a follicle size of circa 3 mm. **e** Vitelline follicles at the F4 stage (4–8 mm). ZPD is a minor IPVL component and is synthesized by the granulosa cells. At this follicular stage, ZPD begins incorporating into the extracellular space. Signals can no longer be obtained from either ZP2 or ZP4. With follicular growth, the perivitelline space becomes tighter and the cytoplasmic processes become shorter. **f** Schema of egg coats after ovulation. Contrary to the ZP in mammals, the ovum does not hatch out of the IPVL. Instead, the ovum becomes covered by the outer perivitelline layer (OPVL) as the innermost chalaziferous layer and the chalazae, synthesized by the infundibulum. After accretion of the egg white in the magnum, the isthmus builds the inner and outer eggshell membranes, forming the air chamber at the blunt egg pole. During passage through the uterus, the calcium eggshell with a thin cuticula is constructed.

For antigen localization, the ABC technique (streptavidin-biotin horseradish peroxidase complex; Dako) was used. The sections were slightly counterstained in hematoxylin for 30 s, dehydrated in a graded series of ethanol, and mounted in Eukitt® (Riedel de Haen, Seelze, Germany).

Control samples, which were obtained by either replacing the primary antibody with buffer or nonimmune serum or incubating with 3, 3′-diaminobenzidine reagent (to exclude the possibility of nonsuppressed endogenous peroxidase activity), were consistently negative in follicles of all sizes and demonstrated the specificity of the immunohistochemical reactions. Images were captured with a Leica Labo-Lux® microscope equipped with a Zeiss Axiocam® camera (Zeiss, Munich, Germany).

Using light microscopical observation, quail follicles of different maturation stages could be classified. In this study, we used the classification of follicles proposed by Sasanami et al. [2004] (table 1). The maximal follicular diameter of the follicles investigated was measured using ImageJ.

Transmission Electron Microscopy

Immediately after the removal of small pieces (1-mm side length) of quail ovary, samples of the IPVL were taken from follicles of the previtelline and vitelline stages. These IPVL samples were subsequently cut and fixed by immersion in Karnovsky's solution (2.5% glutaraldehyde and 2% paraformaldehyde) for 24 h at 4°C. Afterwards, the sections were treated with 1% osmium tetroxide (Plano, Wetzlar, Germany)/1.5% potassium ferrocyanide (Sigma-Aldrich, Steinheim, Germany), dehydrated in a graded series of ethanol (50, 70, and 90%, and absolute ethanol, 30 min each), and embedded in Polyembed 812 BDMA (Polysciences, Eppelheim, Germany). Semithin sections of 1 μm were cut and stained with Richardson's solution [Richardson et al., 1960].

Ultrathin sections were cut using a diamond knife (Reichert-Labtec, Wolfratshausen, Germany), placed on 150-mesh copper grids (Polysciences), stained with uranyl acetate (Scientific Ltd., Stansted, UK) and lead citrate (Agar Aids, Stansted, UK), and examined under a transmission electron microscope (EM 902; Zeiss).

Table 1. Follicle sizes and allocations in the Japanese quail ovary

Follicle 1 (F1)	≥15 mm
Follicle 2 (F2)	12–15 mm
Follicle 3 (F3)	8–12 mm
Follicle 4 (F4)	4–8 mm
Follicle 5 (F5)	2–4 mm
SYF	1–2 mm
SWF	≤1 mm
Previtelline follicle (PVF)	65–450 μm
Primordial follicle (POF)	40–65 μm

Results

General Observations

The ovary of a laying quail contains oocytes varying in size from about 0.05 mm up to approximately 15 mm in maximum diameter. Generally, small primordial follicles consist of an oocyte surrounded by a single layer of flat granulosa cells sitting on a distinct basement membrane. Primordial follicles are located in the outer cortex of the ovary, usually directly beneath the surface epithelium. Activated primordial follicles increase in size, forming a population of previtelline follicles with a size range from 0.06 to 0.5 mm, followed by small white follicles (SWF; 0.5–1 mm) and small yellow follicles (SYF; 1–2 mm). The latter 3 follicular stages show a follicular wall which consists of a single layer of cuboidal-to-cylindrical granulosa cells. This granulosa layer is surrounded by theca interna and externa, which contribute to the stability of the follicular wall. During the later previtelline stages, special contact zones between the oolemma and the microvilli of the granulosa cells develop and are called zona radiata. In the perivitelline space, within the zona radiata, glycoproteins (ZP1, ZP2, ZP3, ZP4, and ZPD) accumulate in the extracellular space between the granulosa cells and oocyte, forming a compact and continuous layer (about 2 mm thick) that covers the oocyte in the mature follicle. During the last stage of follicular growth, the oocytes incorporate a large amount of yolk. These vitelline follicles, which range from follicular stage 5 (F5; the smallest yellow follicle) to F1 (the largest yellow follicle), have a follicular wall that consists of a flat granulosa layer, well-defined theca layers, and a completed IPVL before ovulation. Follicles prior to ovulation are termed 'preovulatory follicles'.

Immunohistochemical Staining

In this study, we used ZP1 as an example of a late (ZP1 material is continuously assembled in the extracellular space in follicles of 3 mm and larger) and ZP2 as an early (ZP2 material is continuously assembled in follicles of circa 0.9 mm) IPVL-incorporating ZP glycoprotein.

ZP1. In primordial follicles, no immunostaining was found. During previtelline follicular stages, circumscribed ZP1-positive material was detected in single-layered granulosa cells (fig. 2a). At first, this material appeared as blotch-like fields within the cytoplasm of the granulosa cells. At a later stage of development, ZP1-positive material was concentrated in the apical cytoplasm of the granulosa cells but was still not released into the extracellular space (fig. 2b). In larger follicles (F5/F4 stage), a continuous ZP1-positive stained acellular layer could be observed for the first time in the extracellular space, lo-

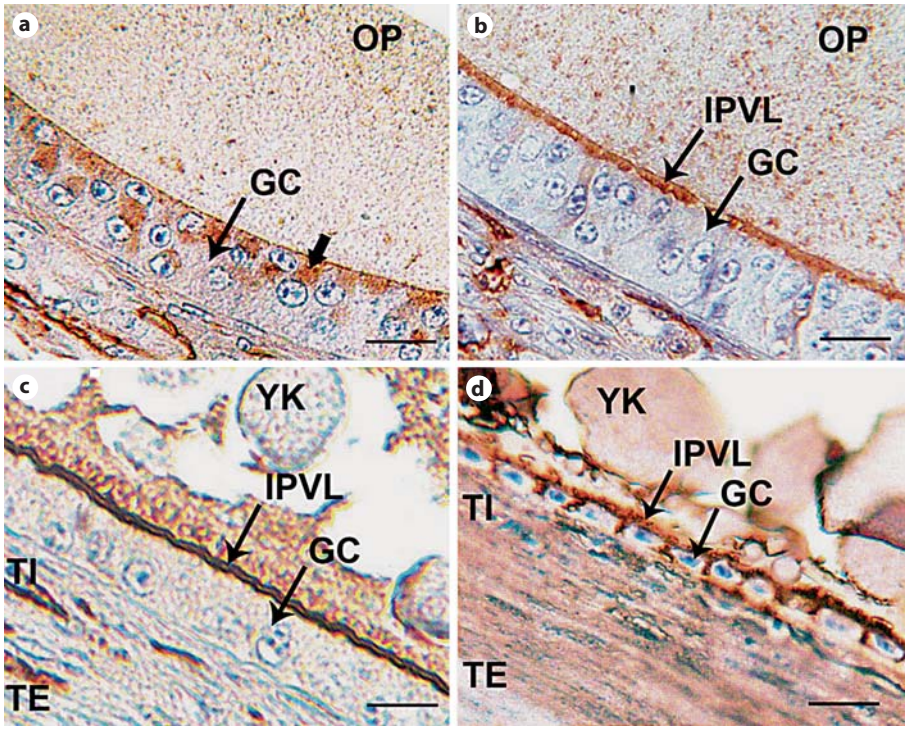

Fig. 2. Immunohistochemical observations of ZP1 localization. **a** Immunostaining of a previtelline follicular wall with an anti-ZP1 antibody. This previtelline follicle (size circa 200 μm) shows immunopositive spherical areas (bold arrow) in various positions in the cytoplasm of the granulosa cells (GC). These areas are assumed to picture ZP1-positive material coming from the bloodstream to pass through the granulosa cells. OP = Ooplasma. Scale bar = 10 μm. **b** Immunostaining of a larger previtelline follicular wall with an anti-ZP1antibody. This previtelline follicle (size circa 400 μm) shows immunopositive glycoprotein material on the apical side of the granulosa cell layer. The ZP1-positive material will contribute to the later IPVL. This material has not yet been incorporated into the extracellular space. Scale bar = 7 μm. **c** Immunostaining of an F4-sized follicular wall with an anti-ZP1 antibody. The IPVL of this F4 follicle (size circa 4,000 μm), already completely located in the extracellular space, first shows a continuous and strong positive reaction with the antibody against ZP1. Some immunopositive staining can also be seen in the blood vessels of the theca externa (TE) and theca interna (TI). YK = Yolk particle. Scale bar = 10 μm. **d** Immunostaining of an F2-sized follicular wall with an anti-ZP1 antibody. In large preovulatory follicles, like the one shown here (size circa 15,000 μm), the ZP1-positive material of the IPVL is located below and between the granulosa cells. Scale bar = 10 μm.

cated between the granulosa cells and the ooplasma membrane of the oocyte (fig. 2c). ZP1 immunopositive staining was also observed in the theca layers, indicating a transport of ZP1 (synthesized in the liver) to the oocyte via the blood stream. At late preovulatory stages, such as F2 follicles, ZP1-positive material is present in comparatively large amounts, forming a distinctly staining layer in the extracellular space. ZP1-positive material is also deposited in the intercellular space between neighboring granulosa cells (fig. 2d).

ZP3 and ZPD, which also incorporate comparatively late into the developing IPVL (ZP3 material is continuously assembled in follicles of 1 mm and larger and ZPD in follicles of 4 mm and larger), showed a similar staining pattern in the growing follicles. Hence, a continuous ZP3-positive layer first appeared at the SYF (1–2 mm) follicular stage, but occasionally some faint ZP3 immunostaining was already seen in the cytoplasm of granulosa cells in previtelline follicles. ZPD, similar to ZP1, was first found as a faint ZPD-positive extracellular line when the follicle could be classified as an F5 follicle. ZPD material accumulates within the IPVL even later than ZP1. Moreover, our immunohistochemical data provided evidence that ZPD is actually the last incorporating glycoprotein of the matrix as it can be demonstrated as a compact structure only in F4-sized and larger follicles. However, in contrast to ZP1 that accumulates between neighboring granulosa cells in the apical cytoplasmic areas of the granulosa cells, this fact is not given for ZP3 or ZPD.

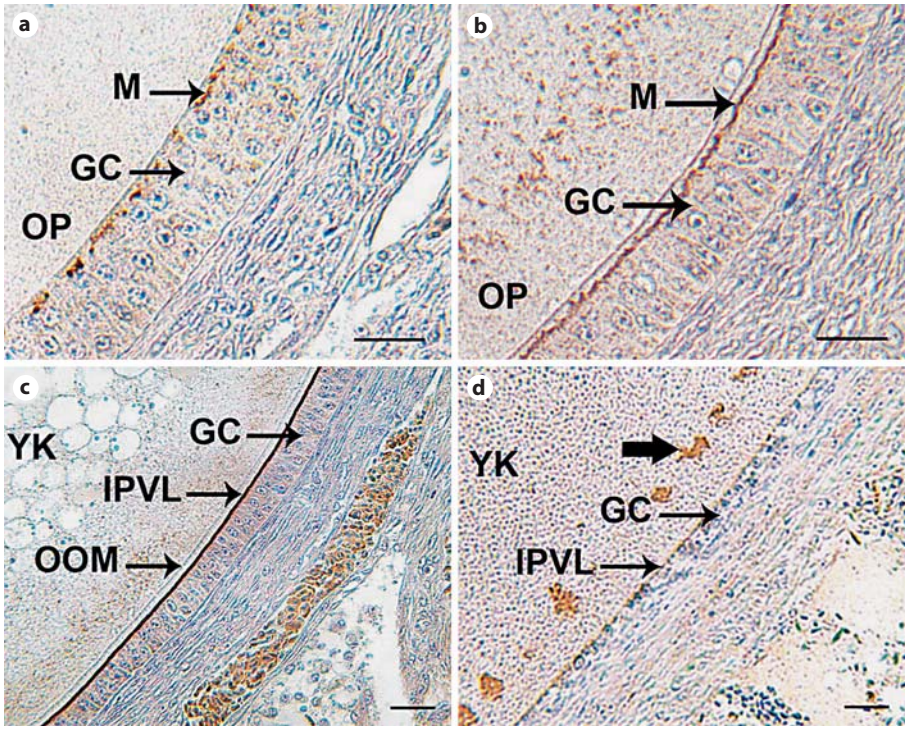

Fig. 3. Immunohistochemical observations of ZP2 localization. **a** Immunostaining of a previtelline follicular wall with an anti-ZP2 antibody. In this previtelline follicle (size circa 200 μm), single granular ZP2-positive spot-like material (M) is located in the extracellular space between the oocyte and the apical side of the granulosa cells (GC) for integration into the future IPVL. OP = Ooplasma. Scale bar = 10 μm. **b** Immunostaining of a small white follicular wall with an anti-ZP2 antibody. ZP2-positive material (M) for the IPVL of this previtelline follicle (size circa 400 μm), although thin and irregular, is assembling in the extracellular space. Some ZP2-positive granules can also be observed in the ooplasm. Scale bar = 10 μm. **c** Immunostaining of a barely small white follicular wall with an anti-ZP2 antibody. This SWF (size circa 900 μm) shows the strongest positive reaction on its IPVL, which is visible as a uniformly stained and continuous line located in the extracellular space between the granulosa cells and the ooplasma membrane (OOM) of the oocyte. YK = Yolk particles. Scale bar = 25 μm. **d** Immunostaining of an F5 follicular wall with an anti-ZP2 antibody. This F5 follicle (size circa 3,000 μm) shows a faint reaction with the ZP2 antibody at its IPVL at the most. The immunostaining of ZP2 actually becomes weaker with increasing follicular size. Only detached ooplasmic cortical contents (arrow) show immunopositive staining with this antibody. Scale bar = 50 μm.

ZP2. Regarding the early incorporating ZP glycoproteins (ZP2 and ZP4), immunopositive ZP2 material could not be found in the primordial follicles. In previtelline follicles, distinct dot-like immunopositive staining of ZP2 was found in the extracellular space adjacent to the apical plasma membrane of the granulosa cells (fig. 3a). Whereas primordial follicles did not contain any ZP2-positive material, a thin and ragged but coherent ZP2-positive layer was observed at the previtelline follicle stage, accumulating between the granulosa cell layer and the oocyte in the extracellular space (fig. 3b). In the ooplasm, many ZP2-positive granules were observed, which may display ZP2 material and contribute to the forming IPVL. At the later SWF stage (0.9 mm), the ZP2 layer appeared as a smooth, continuous, and well-defined acellular layer in the extracellular space (fig. 3c). ZP2-immunopositive granules were no longer found in the ooplasm at this stage. In preovulatory follicles (F5), the immunopositive staining of ZP2 decreased and appeared only faintly (fig. 3d). ZP2 immunopositive staining ultimately disappeared with further oocyte growth. Some ZP2-positive circumscribed areas were occasionally observed in the yolk.

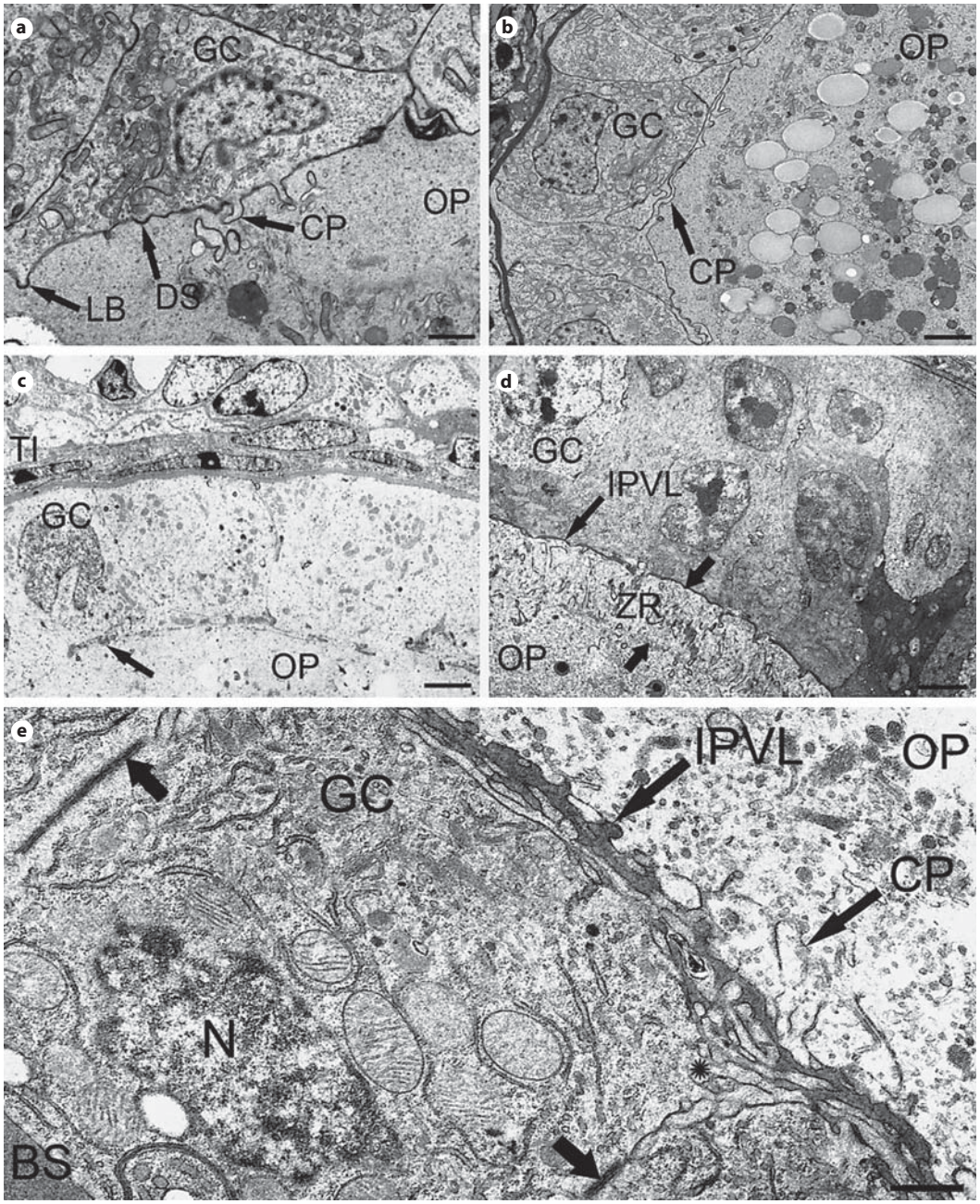

Fig. 4. Ultrastructural observations with transmission electron microscope. **a** Ultrastructure of a primordial follicle wall. Flat granulosa cells (GC) show desmosomes (DS), lining bodies (LB), and cytoplasmic processes (CP) on their apical side as connections between themselves and the oocyte membrane. OP = Ooplasma. Scale bar = 2.5 μm. **b** Ultrastructure of an early previtelline follicle wall. The isoprismatic granulosa cells of an early previtelline follicle wall often show longer cytoplasmic processes than seen in primordial follicles. Scale bar = 3 μm. **c** Ultrastructure of a previtelline follicle wall. The apical side of the granulosa cells shows some dark accumulation of a substance which could probably be identified as ZP material for later incorporation into the IPVL. TI = Theca interna. Scale bar = 3 μm. **d** Ultrastructure of a late previtelline follicle wall. In this late previtelline follicle, an articulate zona radiata (ZR; between the arrows) has been built between the granulosa cells and the oocyte, consisting mainly of their long cytoplasmic processes (which interlock each other) and a thin developing IPVL. Scale bar = 3 μm. **e** Ultrastructure of a vitelline follicle wall circa 25 h prior to ovulation. The IPVL has formed its 3-dimensional network structure in the extracellular space between the granulosa cells and the oocyte. Some of the ZP material could be found at the lateral granulosa cell borders in the intercellular space (asterisk). The demonstrated granulosa cell shows tight junctions (small arrows) as the connection to each neighboring cell. BS = Basal lamina; N = nucleus. Scale bar = 1 μm.

The staining pattern for ZP4 during follicular development was very similar to that of ZP2 with anti-ZP4 antiserum (data not shown). Neither ZP2 nor ZP4 showed any immunopositive staining in the theca layers.

Ultrastructural Observations

In primordial follicles, flat granulosa cells exhibited cytoplasmic processes on their apical side that were connected with the ooplasma membrane via gap junctions and desmosomes (fig. 4a). Lining bodies could also be found. At an early previtelline stage, granulosa cells were isoprismatic and their cytoplasmic processes appeared elongated (fig. 4b). Gap junctions were found between the plasma membrane of the oocyte and granulosa cells. In the ooplasma, a distinct Balbiani body occurred which consisted of mitochondria of crista type, lipid vacuoles and other vacuoles with a content of varying electron density, cisterns of rough and smooth endoplasmic reticulum, dense core particles, and electron-dense bodies. The apical granulosa cell membrane displayed no particular cellular specialization at this follicular stage. At a later previtelline follicular stage, medium electron dense material accumulated in the cytoplasm of the granulosa cells, especially in the apical cell areas that were directed towards the growing oocyte (fig. 4c). In follicles in the previtelline-to-SWF stage, the zona radiata was beginning to form; this consisted of long cytoplasmic processes extending from the granulosa cells and long microvilli of the oocyte, which extensively interdigitate (fig. 4d). Electron-dense material was also beginning to accumulate in the zona radiata and appeared especially concentrated directly above the granulosa cells.

In preovulatory follicular stages, the IPVL glycoproteins appeared fully assembled and formed a 2-μm-thick porous matrix located in the extracellular space between the granulosa cells and the cell membrane of the oocyte (fig. 4e). This matrix appeared to be composed of an open meshwork of branching, electron-dense fibers whose axes were orientated tangentially to the oocyte surface. The zona radiata was withdrawn. At the lateral aspects of neighboring granulosa cells, the intercellular space appeared widened and filled with a moderate amount of electron-dense material.

Discussion

The oocyte of several vertebrate classes (amphibians, reptiles, fishes, birds, and mammals) and of invertebrates (ascidians and molluscs) is surrounded by an extracellular coat consisting of a small number of glycoproteins that show a comparatively high homology in their amino acid composition. These glycoproteins were probably derived from a common ancestral gene and conserved for several hundred million years of evolution [Wassarman, 2008]. Common features of these glycoproteins are an N-terminal signal sequence, a ZP domain, and usually multiple copies of the N-terminal subdomain of the ZP domain, which may play a role in the polymerization of these proteins into supramolecular fibrils or matrices. These glycoproteins also possess a C-terminal propeptide with a consensus furin cleavage site, N- and/or C-terminal transmembrane domain(s), and sometimes a trefoil domain upstream of the ZP domain [Jovine et al., 2002, 2005; Callebaut et al., 2007]. These ZP glycoproteins are synthesized as precursor polypeptides whose N-terminal signal sequence and C-terminal propeptide have been removed, which is imperative for their incorporation into the ZP [Litscher and Wassarman, 2007].

The mouse ZP has been the preferred model for studies of the ZP structure and fundamental understanding of sperm-oocyte interactions for a long time. Contrary to the mouse, in which all zona proteins are synthesized by the growing oocytes [Epifano et al., 1995], granulosa cells in avian species significantly contribute to the IPVL, the avian homolog of the mammalian ZP. Moreover, in domestic animals and humans, both the oocyte and granulosa cells contribute to the formation of the ZP [Lefievre et al., 2004; Goudet et al., 2008].

In the present immunohistochemical and ultrastructural study, we confirmed and extended the previous observations [Pan et al., 2001; Sasanami et al., 2003; Sato et al., 2009; Kinoshita et al., 2010; Serizawa et al., 2010] that glycoproteins in the IPVL of the quail, which originate from several sources [Kinoshita et al., 2010], are deposited in a strict spatiotemporal pattern during folliculogenesis. The immunostaining of IPVL proteins ZP2 and ZP4, which are deposited relatively early on during folliculogenesis, appears attenuated in the more mature follicles. Using an RNase protection assay and in situ hybridization, Kinoshita et al. [2010] and Serizawa et al. [2010] demonstrated that the expression level of ZP2 was highest in SWF follicular stages and strongly decreased during oocyte growth. Further, mRNA for these 2 zona proteins was exclusively expressed in the oocyte. In the most mature follicles (F3, F2, and F1), no signal for ZP2 and ZP4 mRNA could be demonstrated [Kinoshita et al., 2010; Serizawa et al., 2010]. These findings are in good agreement with the immunohistochemical results obtained in our study. We observed that the immunostain-

ing for ZP2 and ZP4 was most distinct in follicles 0.9 mm in size and then became continuously weaker with further follicular growth. We assume that, due to early secretion and incorporation into the developing IPVL, ZP2 and ZP4 build a type of prematrix around the oocyte of early follicles in which other ZP glycoproteins are integrated during later follicular development to establish the mature IPVL. The incorporation of other glycoprotein species into the IPVL (ZP1, ZP3, and ZPD) may mask the epitopes of ZP2 and ZP4, which leads to reductions in immunostaining of these glycoproteins in the IPVL.

ZP1, which is an important glycoprotein of the more mature IPVL of the quail, is synthesized in the liver under the control of estrogens. It is transported to the oocyte via the blood stream and deposited into the IPVL [Sasanami et al., 2003]. Using Western blots, it has been shown in the quail that ZP1 quantitatively contributes the largest amount of glycoprotein to the IPVL of F1 follicles [Sasanami et al., 2004]. Furthermore, we found that ZP1 accumulates in considerable amounts between neighboring granulosa cells of preovulatory follicles. This accumulation may enhance the stability of the IVPL.

In contrast, ZP3 is synthesized by the granulosa cells [Pan et al., 2001]. However, both ZP1 and ZP3 are major constituents of the IPVL. Using Western blotting and SDS-PAGE, ZP1 was first detected in F4-sized follicles and ZP3 in SYF-sized follicles by Sasanami et al. [2004]. In our immunohistochemical study, we confirmed these findings for both glycoproteins.

The glycoprotein that is incorporated the latest into the IPVL of the quail is ZPD, and it is considered to be connected to the IPVL only loosely. Our findings confirm the results of Sato et al. [2009], showing immunopositive staining on the apical side of the granulosa cells. ZPD is not incorporated into the IPVL in follicles before the F5/F4 stage. However, positive staining could be seen earlier in the granulosa cells. Results of earlier in situ hybridization studies [Sato et al., 2009] have demonstrated the occurrence of ZPD mRNA in granulosa cells. These data together with our results lend support to the hypothesis that these cells are the source of ZPD.

In mice, ZP3 and ZP2 serve as primary and secondary sperm receptors during fertilization, while ZP3 is involved in the acrosome reaction [Wassarman, 1999, 2005]. In chickens, spermatozoa bind to ZP1 and ZP3 [Bausek et al., 2004]. In quails, monomeric and dimeric ZP1 induces the exocytosis of the acrosomal content [Sasanami et al., 2007] and plays an important role in sperm-IPVL interaction. However, because of the equal distribution of ZP1, our results do not support the conclusion of Bramwell and Howarth [1992] that spermatozoa preferentially hydrolyze a special region of the IPVL that overlies the animal pole and that this region contains an especially high number of receptors for spermatozoa.

Immunohistochemical and glycohistochemical studies of the mammalian ZP [Sinowatz et al., 2001] have shown that different zones of varying staining intensities exist, which suggest differences in the localization of the ZP glycoproteins within the zona. Our ultrastructural and immunohistochemical results demonstrate a more homogenous distribution of the ZP glycoproteins in the IPVL of the quail. ZP1 and ZP3, which both may serve as sperm receptors, do not show zonation but appear to be equally distributed throughout the IPVL, recognized by its uniform staining quality when assembled completely. Our ultrastructural data show that the mature IPVL is composed of an open meshwork of branching, electron-dense fibers whose axes are orientated tangentially to the oocyte surface. Apart from the granulosa cell processes, the interstices are filled with homogenous material. This homogenous distribution pattern of the glycoproteins of the IPVL is confirmed by our immunohistochemical data which demonstrate that no preferred localization of any of the ZP glycoproteins occurs within the IPVL and that no distinct zonation of the egg coat, as seen in several mammalian species, exists.

In conclusion, immunohistochemical and ultrastructural characterization demonstrates that the quail IPVL (as a paradigm of an avian egg envelope) consists of 5 ZP glycoproteins that are incorporated into the IPVL of growing follicles at distinct time points. Contrary to the situation in mammals, not only glycoproteins from the oocyte (ZP2 and ZP4) and from the granulosa cells (ZP3 and ZPD) but also ZP proteins synthesized in the liver (ZP1) significantly contribute to the IPVL. This diversity in the source of the glycoproteins may be due to the fact that the formation of the IPVL, covering a huge polylecithal avian oocyte, needs considerably more glycoprotein material compared to the ZP of a small oligolecithal mammalian egg.

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