Identification of Boar Sperm Ligands Bound to the Protein (pZP1) of Pig Zona Pellucida

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Abstract: *In previous studies we found that pig zona pellucida glycoprotein pZP1 may act as the secondary sperm receptor during penetration into the zona pellucida. In this study, a recombinant pZP1(1-198) protein (r-pZP1) was produced to examine the direct binding of pZP1 to spermatozoa. When boar spermatozoa were co-cultured with pig cumulus oophorus in TC199 medium containing r-pZP1 proteins, r-pZP1 bound to the spermatozoa invaded the cumulus oohorus. The binding site was in the acrosomal region, but no sperm binding of r-pZP1 was observed when they were incubated in the medium without cumulus oophorus. This suggests that the cumulus cells played a role in exposing the ligand molecules for r-pZP1 in the acrosomal region. When boar spermatozoa were incubated with pig cumulus-oocyte complex in the medium containing r-pZP1, r-pZP1 bound to the spermatozoa adhered to the zona pellucida in the postnuclear region and the tail. r-pZP1 also bound to human spermatozoa pretreated with Ca^{++} ionophore A23187 and the binding site migrated from the equatorial region to the tail during incubation. Western blot analysis with crude extracts of boar spermatozoa revealed that three molecules, Mr 55K, Mr 40K and Mr 25K, reacted to r-pZP1.*

Key words: *Secondary sperm receptor, Pig zona pellucida, pZP1, Sperm-ZP interaction.*

The zona pellucida is an extra cellular matrix surrounding mammalian eggs and consists of three major glycoproteins. It plays an important role in sperm recognition, activation, penetration and the prevention of polyspermy [1, 2]. In mice, mZP3 is known to act as the primary sperm receptor and possess the acrosome reaction-inducing activity. mZP2 is associated with the secondary sperm binding to the zona pellucida during zona penetration. mZP1 is a structural component that maintains the filamentous structure of the zona pellucida by interconnecting with mZP3 and mZP2 heterodimers [2–4]. Pig zona pellucida is composed of three glycoprotein families, pZP1, pZP3α and pZP3β. pZP3α was shown to have a sperm adhesion activity as the primary sperm receptor [5]. In mice, three molecules of galactosyl transferase, sp56 and tyrosine kinase (Mr 96K) were reported to function as a ligand for mZP3 [6–8]. On the other hand, PH20 in guinea-pigs, SP10 in humans and a proacrosin-like molecule of Mr 38K in boars were reported to be a ligand molecule for the secondary sperm receptor [9–11]. Our previous study showed that the DNA sequence of pZP1 was homologous to that of mZP2 and suggested that pZP1 may function as a secondary sperm receptor similar to mZP2 [12, 13]. In the present study, we produced a pZP1 (1–198) protein by using recombinant technology and examined for the binding activity of the recombinant protein to boar spermatozoa by immunofluorescent staining and immunoblotting tests. The results showed that the recombinant pZP1 (r-pZP1) bound only to the capacitated and acrosome-reacted spermatozoa and that three components of the spermatozoa with Mr 55K, Mr 40K and Mr 25K bound to r-pZP1.

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Materials and Methods

(1) Preparation of r-pZP1

In this study, the cDNA corresponding to the NH_2-terminal 1–198 amino acid region, which does not contain a signal peptide, was inserted in a expression vector (pET21b, Novagen) (Fig. 1). The cDNA was prepared from the four overlapping cDNA fragments(I–IV) coding

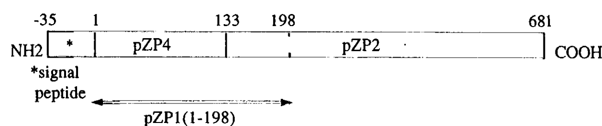

Fig. 1. Structure of pZP1 core protein revealed by cDNA cloning. The NH_2-terminal 35-amino acid sequence corresponds to a signal peptide. pZP1 is divided into pZP4 and pZP2 between amino acids 133 and 134. The pZP1(1–198) peptide was produced by recombinant technology in the present study.

for pZP1 (1–77), pZP1 (53–86), pZP1 (70–110) and pZP1 (94–198) respectively. These fragments were connected at the Nde I site (between fragments I and II), at the Bal I site (between fragments II and III) and at the Bam HI site (between fragments III and IV). The constructed vector pET21b-pZP1 (198) was transfected in *E. coli* (BL21). pZP1-producing cells were cloned by colony hybridization with a monoclonal antibody (MAb-5H4)which could recognize the epitope on pZP1 (50–67) peptide [15]. The recombinant pZP1 (r-pZP1) producing clone was cultivated on a large scale and induced the expression by IPTG. For preparation of r-pZP1, the proliferated bacteria were solubilized with 1% Triton-X and lysozyme of 200 μg/ml followed by centrifugation at 20,000 x g for 15 min at 4°C. The target protein was contained in the pellet as an inclusion body. The pellet was solubilized with 6 M urea at 4°C for 60 min and then the r-pZP1 protein was purified by column affinity chromatography with pET His-Tag™ systems (Novagen).

(2) Immunofluorescent staining of spermatozoa

The cumulus masses were collected from pig ovarian follicles and cultured overnight in TC 199 medium supplemented with 10% FCS, 10IU PMS and 10IU HCG for maturation. The cumulus masses with or without oocytes were used as a cumulus-oocyte complex and cumulus oophorus, respectively. Boar spermatozoa were cryopreserved in liquid N_2 as described before [14]. A frozen sperm tablet was thawed in TC199 medium and centrifuged at 600 xg for 5 min. The washed sperm pellet was overlaid with TC199 medium supplemented with 10% fetal calf serum and 5mM caffeine and then incubated in 5% CO_2 in air for 10 min. Swim-up spermatozoa were transferred to the fresh medium containing r-pZP1 (10 μg/ml) and co-cultured with pig cumulus oophorus or cumulus-oocyte complex. After incubation for 2 hr, the spermatozoa which invaded the cumulus oophorus or were lightly bound to the zona pellucida of the oocytes were collected by pipetting, and this was followed by washing with PBS. The washed spermatozoa were placed on a slide glass and air dried. After fixation with methanol, the sample was treated with a monoclonal antibody (MAb-5H4) [15] for 30 min. After washing, the sample was subjected to the second reaction with FITC labeled anti mouse IgG (Cappel).

Human spermatozoa were obtained from a fertile man. After liquefaction, they were washed with BWW-BSA three times and then incubated for 18 hr for capacitation. Swim-up spermatozoa were transferred to the fresh medium and were treated with 10 μM of Ca^{++} ionophore A23187 (Sigma) for 15 min. After centrifugation, the spermatozoa were resuspended and cultured in the new medium containing r-pZP1 (10 μg/ml). Samples of the human spermatozoa were taken at 2 h, 6 h and 18 h intervals and were processed for immunofluorescent staining as described above. Human cumulus oophorus was obtained from patients undergoing IVF-ER therapy under informed consent.

Spermatozoa of other experimental animals (rabbits, guinea pigs, mice) were collected from epididymides. They were treated with Ca^{++} ionophore A23187 and followed by incubation in an adequate medium containing r-pZP1 for 18 h. The binding of r-pZP1 to the spermatozoa was detected by immunofluorescent staining.

(3) SDS-PAGE and western blotting

Boar epididymal spermatozoa were suspended in the medium containing of 10% glycerol, 3% CH_3COOH and 5 mM benzamidine at a concentration of 1×10^8 cells/ml. The sperm suspension was incubated at 4°C for 2 h. After centrifugation at 5,900 x g for 10 min, the supernatant was dialyzed to 5 mM NH_4HCO_3 and used as an acid extract of boar spermatozoa. The sperm pellets were washed with PBS by centrifugation and were resuspended in 6 M urea solution containing 0.5% mercaptoethanol. The sperm suspension was incubated at 4°C for 18 h and centrifuged at 22,000 x g for 15 min. The supernatant was dialyzed to 5 mM NH_4HCO_3 and used as a urea extract of boar spermatozoa. SDS-PAGE and western blotting were carried out as shown in the previous report [15]. Briefly, acid extracts and urea extracts were applied on the gel in the amount of 50 μg protein per lane. The blotted PVDF membrane was blocked with 3% BSA in Tris-HCl buffered saline (TBS) for 15 min and was incubated in r-pZP1 (10 μg/ml) for 18 h at 4°C. After washing with TBS three times, the membrane was treated with MAb-5H4 and followed by reaction with peroxidase conjugated anti-mouse IgG (1:1,000) (Bio-Rad). Positive staining bands were visualized by reaction with 4-chloro-1-naphthol and H_2O_2.

(4) Analysis of amino acid sequence

Acid extracts from boar spermatozoa were applied to SDS-PAGE and stained with 0.1% coomasie brilliant blue (CBB-R). Proteins corresponding to the positive staining bands in the western blotting were extracted from the gel for micro-sequencing (Applied Biosystems 477A).

Results

When boar spermatozoa were incubated in the medium containing r-pZP1 proteins, r-pZP1 did not bind to the spermatozoa (Fig. 2-a), but when they were co-cultured with the cumulus oophorus in the incubation medium, r-pZP1 bound to the spermatozoa that invaded the cumulus oophorus and attached to the cumulus cells. The binding site was mainly in the acrosomal region (Fig. 2-b). The spermatozoa that did not invade the cumulus oophorus were not reactive to r-pZP1. The cumulus cells might have induced some changes on the acrosome so as to expose the binding ligand for r-pZP1. When cumulus-oocyte complex was added to the incubation medium, r-pZP1 bound to the spermatozoa which once attached to the zona pellucida and then dispersed from it by pipetting. The binding site was in the postnuclear region and the tail (Fig. 2-c), which was different from the binding site of the spermatozoa incubated with cumulus oophorus. Prolonged incubation (18 h) of the boar spermatozoa with the cumulus oophorus increased the number of positively stained spermatozoa, but the binding site of r-pZP1 was restricted to the acrosome region. The acrosome staining of the spermatozoa attached to the cumulus cells had not altered even after 18 h-incubation. When boar spermatozoa were incubated with denuded oocytes, r-pZP1 did not bind to the spermatozoa which adhered to the zona pellucida within 2 h incubation, but when the incubation time was prolonged for 18 h, r-pZP1 was shown to bind to the postnuclear region and the tail, similar to the pattern in Fig. 2-c.

In the experiments with human spermatozoa, r-pZP1 did not bind to the spermatozoa incubated in the medium containing r-pZP1 for 2 h (Fig. 3-a), but when human spermatozoa were pre-treated with Ca^{++} ionophore A23187 for 15 min and were then incubated in the medium containing r-pZP1 for 2 h, r-pZP1 bound to the equatorial segment of the spermatozoa (Fig. 3-b). After 6 h of incubation, some spermatozoa were stained in the postnuclear region (Fig. 3-c) and after further incubation for 18 h, the proportion of spermatozoa stained in the tail was increased (Fig. 3-d). The spermatozoa not treated with A23187 did not show any positive reaction even after 18 h of incubation. When human spermatozoa were incubated with human cumu-

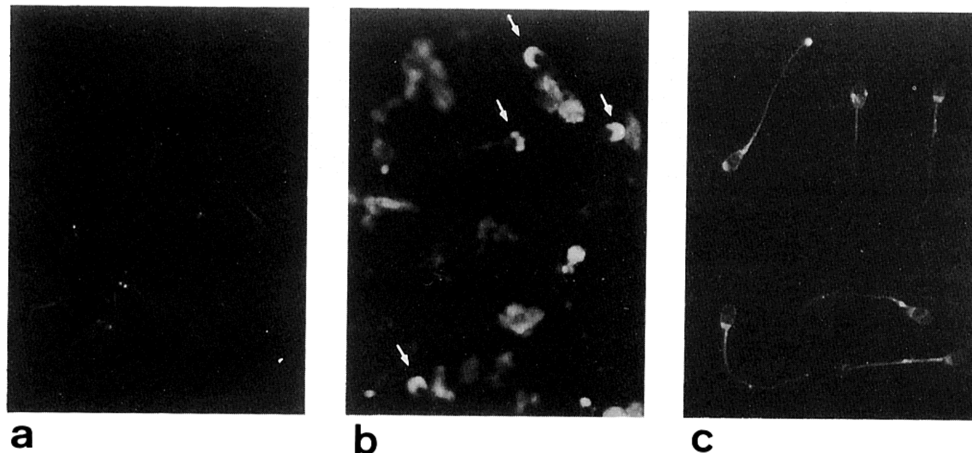

Fig. 2. Indirect immunofluorescent staining of boar spermatozoa with r-pZP1. When boar spermatozoa were incubated only in the medium containing r-pZP1 for 2 h, r-pZP1 did not bind to the spermatozoa (a), but when they were incubated with the cumulus oophorus in the medium containing r-pZP1, r-pZP1 bound to the spermatozoa that invaded the cumulus and attached to the cumulus cells, as indicated with white arrows (b). When they were incubated with cumulus-oocyte complex in the medium containing r-pZP1, r-pZP1 reacted with the spermatozoa which adhered to the zona pellucida in the postnuclear part of the tail regions (c).

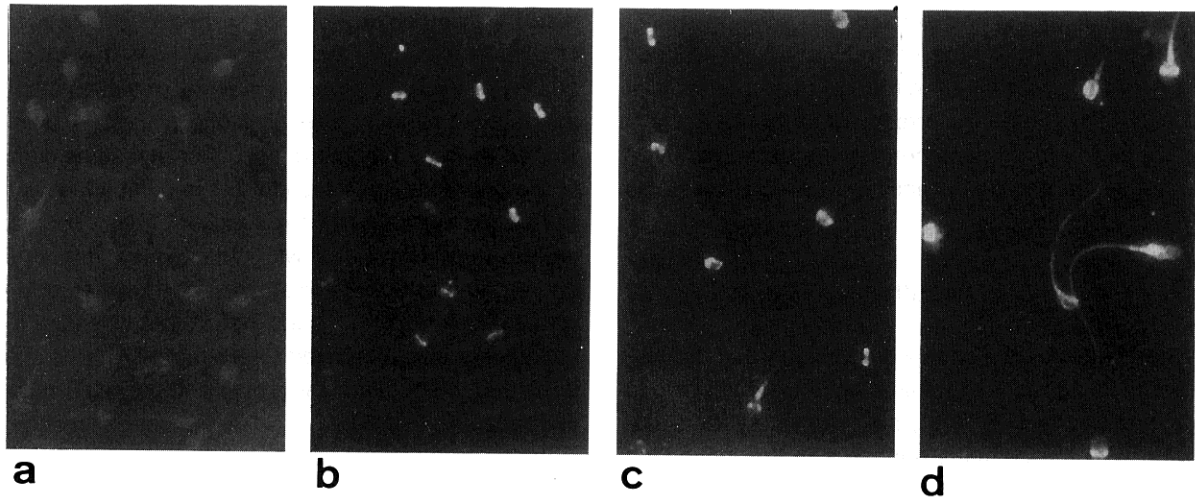

Fig. 3. Indirect immunofluorescent staining of human spermatozoa with r-pZP1. When human spermatozoa were incubated in the medium containing r-pZP1 for 2 h, no positive staining was observed (a), but when they were treated with Ca^{++} ionophore A23187 and then incubated in the medium containing r-pZP1 for 2 h, r-pZP1 bound to the spermatozoa in the equatorial segment (b). When the A23187-treated human spermatozoa were incubated with r-pZP1 for 6 h, some spermatozoa were stained in the postnuclear region (c), and after further incubation with r-pZP1 for 18 h, the spermatozoa were stained in the postnuclear to tail regions (d).

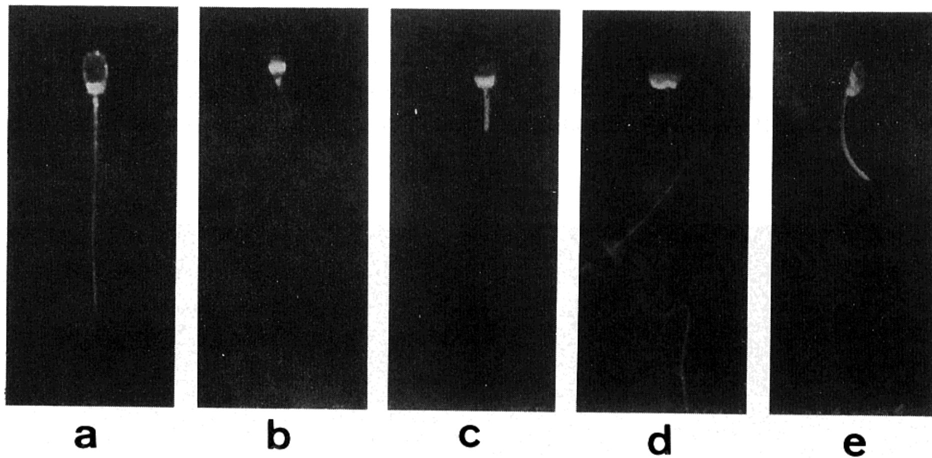

Fig. 4. Indirect immunofluorescent staining with r-pZP1 of spermatozoa from five different species. All five species showed a positive reaction in the post acrosomal region of the tail. Boar (a) spermatozoa were co-incubated with the cumulus oophorus for 18 h. Human (b), rabbit (c), guinea-pig (d) and mice (e) spermatozoa were treated with Ca^{++} ionophore and followed by subsequent incubation for 18 h.

lus oophorus instead of Ca^{++} ionophore treatment, r-pZP1 bound to the equatorial region of the spermatozoa that invaded the cumulus oophorus.

Figure 4 shows the summarized results of the binding of r-pZP1 to spermatozoa from boars (a), humans (b), rabbits (c), guinea-pigs (d) and mice (e). r-pZP1 bound to the spermatozoa from all species in the postnuclear region and the tail.

The acid extract of boar spermatozoa showed two major protein bands (Mr 55K, Mr 40K) in SDS-PAGE (Fig. 5-a). Both protein bands reacted with r-pZP1 in western blotting (Fig. 5-b). The NH2-terminal amino acid sequence of the Mr 55K was determined to be VVGGMSAEPG. The sequence was identical to the

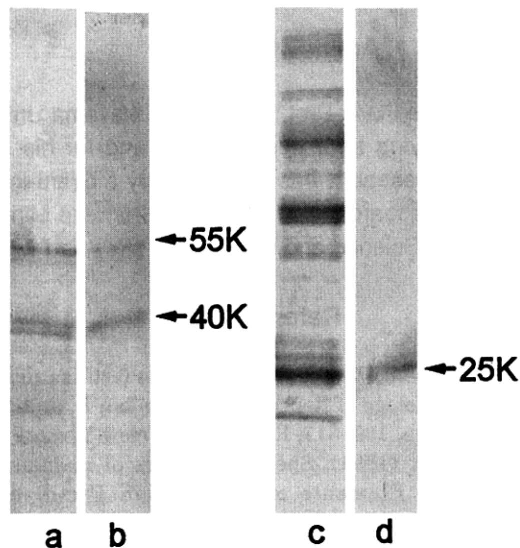

Fig. 5. SDS-PAGE and western blotting of boar sperm extracts with r-pZP1. r-pZP1 reacted with Mr 55K and Mr 40K protein bands of the acid extract (a), and reacted with a Mr 25K protein band of urea extract (c). a, c: western blotting; b. d: protein staining with CBB-R.

NH_2-terminal amino acid sequence of boar sperm proacrosin. In contrast, the NH_2-terminal amino acid sequence of the Mr 40K was determined to be SDVFTQYIGD. This sequence showed no significant similarity to any other proteins by homology search with DNASIS (April, 1996). Fig. 5-c shows protein bands separated in SDS-PAGE of the urea extract of boar spermatozoa. A single band of Mr 25K reacted with r-pZP1 in western blotting (Fig. 5-d).

Discussion

In the study presented here, a pig zona pellucida component (r-pZP1) was synthesized by recombinant technology and used for examination of its capacity to bind to the spermatozoa.

The acrosome reaction of spermatozoa is generally believed to be induced by a glycoprotein of the zona pellucida, such as mZP3 in mice [3]. Acrosome-reacted spermatozoa lose the acrosomal plasma membrane bearing the ligand for the primary sperm receptor on the zona pellucida, but they still remain adhered to the zona pellucida. During the subsequent process of sperm penetration, the repeated binding and hydrolysis occurs between ligands and secondary sperm receptors. In mice, mZP2 is proposed to be a candidate for the secondary sperm receptor on the zona pellucida [2]. pZP1 is also assumed to be the secondary sperm receptor in pigs because of its DNA sequence homology to mZP2 [12, 13].

In the present experiments, r-pZP1 did not bind to boar spermatozoa incubated only in the medium or not invading the cumulus oophorus, but r-pZP1 bound to the spermatozoa attached to the cumulus cells or adhered to the zona pellucida, although the binding sites were different. This suggests that boar spermatozoa could produce or expose the binding molecules to r-pZP1 during the co-incubation with cumulus oophorus. When the spermatozoa attached to the zona pellucida in the cumulus-oocyte complex, the binding site of r-pZP1 moved from the acrosomal region to the postnuclear, midpiece and tail regions. This observation indicated the possibility that the expression of the r-pZP1 binding ligand was induced by the attachment of spermatozoa to the cumulus cells, and the migration of the molecule was triggered by the adherence of spermatozoa to the zona pellucida. The boar spermatozoa that adhered to the zona pellucida without interaction with the cumulus cells did not react with r-pZP1 within 2 h-incubation, but they became reactive with r-pZP1 in the postnuclear and tail regions after 18 h-incubation. This suggested that the binding molecules might be newly exposed on the postnuclear region and the tail or that the molecules might migrate to the posterior regions from the acrosome.

In the experiments with human spermatozoa treated with Ca^{++} ionophore A23187, they did not show any reaction with r-pZP1 immediately after the treatment, but they gradually came to express the ligand for r-pZP1 during the incubation. These results suggest that the binding of r-pZP1 to the spermatozoa is not the primary event but rather associated with the secondary event of the interaction between the spermatozoa and the zona pellucida. The observation that r-pZP1 was reactive to the acrosome reaction-induced spermatozoa from five different species also indicates that pZP1 may be a secondary sperm receptor, because the primary binding between the spermatozoa and the zona pellucida is believed to be a relatively species-specific phenomenon. The site for the ligand expression on the human spermatozoa migrated from the equatorial region to the tail with incubation time. This phenomenon was very similar to that seen in the boar spermatozoa.

It took 18 h for the majority of spermatozoa treated with Ca^{++} ionophore to finish the migration of the ligand molecule to the tail. It is generally believed that Ca^{++}

ionophore-treated spermatozoa can complete the zona penetration in 1–2 h after the treatment. Therefore, in the presence of the zona pellucida in the incubation medium, human spermatozoa may promote more rapid migration of the ligand molecule, similar to the boar spermatozoa. Some molecules on the sperm membrane, such as pH20 [16], rabbit acrosin [17] and SP10 [18] were reported to change their localization during a physiological fertilization process. Our findings on the migration of the r-pZP1 binding site on the spermatozoa were compatible to the previous observations. The results of the present study might indicate that the ligand molecule promotes the penetration of the spermatozoa through the zona pellucida. In this process, the spermatozoa probably acquire the progressing force by migrating the ligand in the head to tail direction.

Western blotting of boar sperm extracts showed that r-pZP1 bound to three different protein bands on SDS-PAGE. One of the bands, Mr 55K, was identified as proacrosin by the NH_2-terminal amino acid sequence analysis. The observation that boar sperm proacrosin interacts with zona pellucida has already been reported in a number of papers [19–21]. In these reports, it was shown that the receptor molecule of the zona pellucida is a carbohydrate moiety of pZP3α. In contrast, the binding of r-pZP1 to the proacrosin was mediated with a peptide molecule as shown in this report, because r-pZP1 does not contain any carbohydrates. Proacrosin is shown to be composed of several domains including the recognition site for pZP3α and the active site for the proteolytic enzyme activity [21]. The binding domain for r-pZP1 is probably different from the binding domain for pZP3α. Immunofluorescent staining with anti-boar proacrosin antiserum showed that boar proacrosin was localized in the acrosomal region and did not migrate to the midpiece or tail during incubation (Data not shown). Therefore, the migration of the r-pZP1 binding site is not due to the proacrosin of Mr 55K in SDS-PAGE. Other protein bands of Mr 40K or Mr 25K would be related to the migration phenomenon.

Recently, Baba *et al.* reported that the sperm from mice carrying a defective acrosin gene can still penetrate the zona pellucida, suggesting that the acrosin is not essential for fertilization [22]. Collectively, a number of molecules and domains on the spermatozoa are associated with sperm penetration through the zona pellucida. One interaction might be compensated for by another one. The molecules of Mr 40K and Mr 25K identified in this study are possibly involved in the secondary sperm binding to the zona pellucida.

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