—Review—

The current perspectives on the mammalian zona pellucida

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Abstract: The mammalian zona pellucida (ZP) is an extracellular matrix that surrounds ovarian oocytes, ovulated eggs, and preimplantation embryos, and it plays several important roles at different stages of reproduction. Newly developed technologies such as transgenic mouse production, database analysis of signal networks, and live-cell imaging have revealed novel findings about the ZP and fertilization. Assisted reproductive technology has also provided new insights into human ZP morphology and function. Recent micromanipulation technologies such as intracytoplasmic sperm injection, are very helpful for treatment of ZP-related infertility. This article describes the current understanding of the following aspects of the mammalian ZP: I) ZP structure, II) ZP functions, III) ZP-related infertility and IV) ZP-based immunocontraceptive vaccines.

Key words: Fertilization, Infertility, Contraception, Assisted reproductive technology, Sperm

Introduction

The zona pellucida (ZP) is an egg coating that is formed around female gametes, and it has long been a popular target for biological and medical research. It acts as a barrier to spermatozoa and finely regulates penetration of a single sperm; the mechanisms underlying these processes and related molecules differ among animal species [1]. A number of studies have shown that the ZP is associated with induction of the sperm acrosome reaction and polyspermy blocking to prevent fertilization by multiple sperm. In particular, monospermic fertilization is necessary to ensure next generation development in all animal species. However, the molecular bases of sperm–egg interaction and polyspermy blocking are not completely clear.

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The human ZP is believed to play several critical roles in human reproduction. Deficiencies in those functions may cause infertility that can be treated using assisted reproductive technology (ART) such as intracytoplasmic sperm injection (ICSI). ZP antigens have been reported to induce self-reactive antibodies that cause infertility [2–4]. Intensive studies have also been conducted to develop ZP contraceptive vaccines. This review describes new aspects of the mammalian ZP in reproductive fields.

I) ZP Structure

1. Biochemical analysis

The human ZP is composed of four glycoproteins (ZP1, ZP2, ZP3, and ZP4), which are each encoded by a single gene (*zp1, zp2, zp3*, and *zp4*, respectively). The mouse ZP contains only three components, because mouse *zp4* is a pseudo-gene caused by stop codons inserted in the *zp4* sequence. Fig. 1 shows the phylogeny of mouse and human zona proteins. A single ancestral gene for the ZP is thought to have diverged into two branches, *zp1/2/4* and *zp3*, early in the evolutionary process. Subsequently, *zp3* diverged into mouse and human *zp3*. The other branch, *zp1/2/4*, diverged into *zp2* and *zp1/4*. *zp1/4* then diverged into *zp1* and *zp4* in humans, but transcriptional *zp4* did not evolve in mice. The ancestral *zp1* diverged into mouse and human *zp1*.

In general, ZP glycoproteins are mainly synthesized by the oocyte during ovarian folliculogenesis. The four glycoproteins in the mammalian ZP are shown as a schematic in Fig. 2. All of these glycoproteins possess ZP domains with similar DNA sequences to each other [5]. The translated polypeptides are sorted as secreting components in vesicles, glycosylated in the Golgi apparatus, and transported to near the cell membrane before secretion. The secretory vesicles contain glycoproteins that partially form steric structures of ZP. Additionally, intra- and intermolecular disulfide bonds constitute complex insoluble polymers after secretion. Although the mechanism is unclear, the secretion occurs intermittently

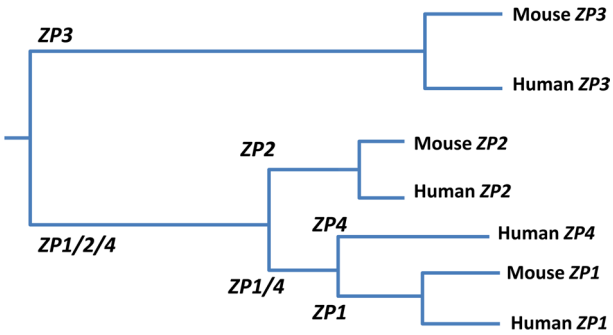

Fig. 1. Phylogeny of mouse and human ZP proteins. A single ancestral gene for ZP is thought to have diverged into two branches, *zp1/2/4* and *zp3*, early in the evolutionary process. Subsequently, *zp3* diverged into mouse and human *zp3*. *zp1/2/4* diverged into *zp2* and *zp1/4*; then, *zp1/4* diverged into *zp1* and *zp4* in humans, but *zp4* did not evolve in mice.

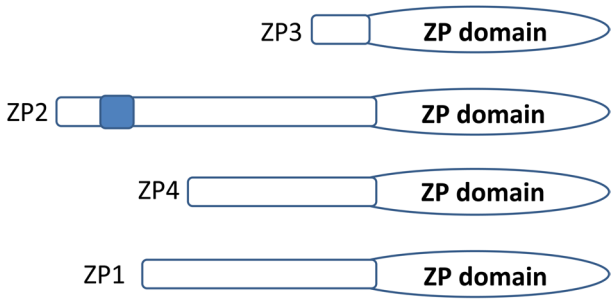

Fig. 2. Schematic drawing of prototypes of ZP1, ZP2, ZP3, and ZP4. All four ZP proteins possess domains that contain homologous sequences at the amino terminus, but their regions differ by size and sequence at the carboxyl terminus. ZP proteins polymerize by intra- and intermolecular disulfide bonds to form the insoluble ZP architecture.

during oocyte growth and is incorporated into only the innermost layer of the ZP [6]. The putative transmembrane domains at the C terminus of ZP2 and ZP3 (called ZP domains) are necessary for incorporation into the ZP already existing outside the oocyte.

Experiments using mice revealed that dormant oocytes in primordial follicles did not express ZP proteins [7]. At the primary follicle stage, with an oocyte surrounded by one layer of cuboidal granulosa cells, the ZP architecture can be observed around the oocyte as a discontinuous layer. In humans, however, ZP glycoproteins seem to be synthesized and stored in primordial oocytes prior to secretion [8]. During follicle/oocyte growth, the ZP becomes thicker until the oocyte matures. The full thickness is approximately 10–15% of the diameter of the ooplasm (7–8 μm in mice and 15–20 μm in humans). In most species, after ovulation, a single spermatozoon penetrates the ZP to complete fertilization. Until implantation, the ZP remains around the developing embryo to protect it during transport in the oviduct and uterus. Details are explained in II-4 of this section.

2. Taxonomic sperm binding to the ZP

Previous studies reported that the ZP only bound to spermatozoa from the same species. Therefore, for a long time it was believed that the ZP possessed the ability to select spermatozoa and avoid fertilization by different species, which indicated that the ZP could recognize sperm species. Thus, the mouse ZP does not bind to human spermatozoa and vice versa. However, this is not the case in combinations of other animal species. Many researchers have made efforts to resolve the molecular mechanisms of species-specific recognition between the ZP and spermatozoa. Thirty years ago, the widely accepted paradigm was that carbohydrate moieties could interact with sperm surface molecules [9]. This hypothesis, however, is not consistent with results produced by recent analysis using transgenic technologies in mice. A portion of the ZP2 protein was proposed to be a sperm-specific binding molecule [10]; in fact, transgenic mice that contain human ZP2 instead of mouse ZP2 can be fertilized by mouse spermatozoa and are fertile.

The putative sperm binding site is not explained by a single molecular interaction, and species recognition by the ZP is thought to be substantially more complicated. It was demonstrated that mouse ZP in which all ZP proteins were replaced with human ZP proteins bound to human spermatozoa but not to mouse spermatozoa, and the resulting transgenic mice were infertile. The critical region of species specificity was found to be located in the C-terminal region of ZP2 [11].

3. Human ZP polymorphisms

Although the human ZP exhibits genetic polymorphisms, most do not affect sperm binding or the fertilizing ability of the oocyte. One study showed that a single nucleotide polymorphism (SNP) affected ZP structure, but fertilizing ability was normal. Furthermore, there was no evidence of any relationship between the occurrence of infertility and particular DNA sequences [12, 13]. Collectively, irregular ZP morphology is probably caused by the local environment of surrounding somatic cells but does not influence the sperm binding and fertilizing abilities of ZP. Abnormal ZP morphologies that our laboratory observed are shown in Fig. 3; Excepting panel D, two

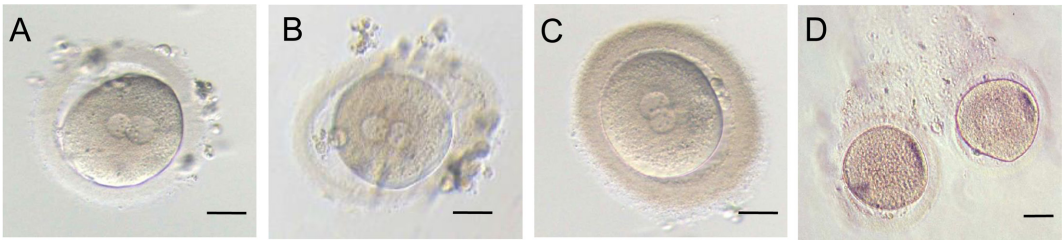

Fig. 3. Human ZPs showing different morphologies observed during IVF treatment. A, normal ZP; B, ellipse-shaped ZP; C, thick and dark ZP; D, abnormal ZP formation. The zygotes in A, B, and C are at the pronucleus stage, which indicates that spermatozoa penetrated the ZP and oolemma. The oocytes in D show no pronuclei, probably because of failed penetration of the ZP by spermatozoa. Bars: 30 μm.

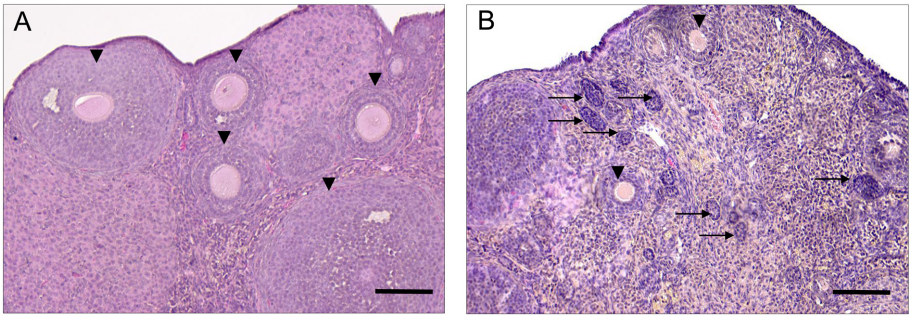

Fig. 4. Ovarian histology of ZP2-knockout mice. A: Wild-type mouse (control). Fully grown oocytes and secondary follicles are present (▼). B: ZP2-knockout mouse. Oocyte diameters are smaller than those of the wild-type mouse (▼). Many pyknotic cells and follicles without oocytes are present (→). Small numbers of oocytes were ovulated as a result of gonadotropin stimulation and fertilized by careful ICSI in B. Both sections were taken from 8-week-old mice, which were of young reproductive age. Bars: 100 μm.

pronuclei derived from egg and sperm can be seen in the oocyte cytoplasm, which provides evidence of sperm penetration of the ZP.

II) ZP Functions

1. Oocyte growth in the ovary

Animal experiments demonstrated that mice knocked out for the genes that encode zona proteins ZP2 and ZP3 were completely infertile [14, 15]. ZP1 knockout mice showed significantly reduced fecundity [16]. Fig. 4 shows the ovarian histology of a ZP2-knockout mouse. A number of small follicles that lacked oocytes were present (Fig. 4B). This indicates that in humans, aberrant expression of ZP proteins by genetic ZP mutations might cause infertility. Case reports described that ZP-free or ZP-fragile oocytes were retrieved from women with recurrent "genuine empty follicle syndrome" (GEFS) [17, 18]. Such ZP abnormalities might arise from mutations in the genes that encode ZP proteins, although careful investigation is needed for definitive diagnosis. However, oocytes with abnormal ZPs were reported to have been fertilized by ICSI and to have led to normal births [19, 20].

2. Induction of the sperm acrosome reaction and interaction of the ZP and sperm

Under physiological conditions, the acrosome reaction, which is a kind of endocytosis of sperm head regions, is necessary for sperm penetration into the ZP in most animal species [21]. Previous studies have shown that capacitated spermatozoa bind to the ZP, which induces the acrosome reaction. It was widely accepted that the molecule that mediated sperm binding and triggered the acrosome reaction was ZP3, which was considered the sperm receptor. However, it was recently reported that, in some spermatozoa, the acrosome reaction completes before attachment to the ZP and that those sperm can penetrate the ZP and fertilize [22]. Similarly, acrosome-reacted spermatozoa collected from the perivitelline space can again penetrate the ZP of another oocyte [23].

These reports rendered the functions of ZP3 ambiguous. Therefore, the molecular basis of gamete recognition at the ZP surface is still to be determined.

Numerous molecules have been proposed as binding candidates at spermatozoa sites. Knockout mouse experiments have shown that there is no molecule that causes null mice to become infertile except for a disintegrin and metalloprotease (ADAM) 3 [24]. In addition, ADAM3 seems to be involved in ZP binding and oviduct transport of spermatozoa. Recently, a molecule named TEX101, which is cleaved by angiotensin-converting enzyme, was revealed to be essential for ADAM3 function [25]. Mysteriously, however, ADAM3 has not been identified as a protein but has only been recognized as DNA and mRNA. Collectively, the acrosome reaction and interactions of the ZP are not entirely explainable by molecular events.

3. Prevention of polyspermy

The contribution of the ZP to prevention of polyspermy differs under in vitro and in vivo conditions. In vitro conditions including those for assisted reproductive technology require a concentration of 5×10^4 /ml spermatozoa for insemination. Many spermatozoa, therefore, bind to the ZP under in vitro conditions, and polyspermy occurs at a higher frequency in vitro. Under in vitro conditions, the ZP definitely plays a role in polyspermy prevention. As a mechanism, it is believed that cortical granules located beneath the oocyte membrane release enzymes that change ZP functions after one spermatozoon has penetrated and fused to the oocyte membrane. This is called the “zona reaction” or the “cortical reaction”. Additional spermatozoa are blocked from penetrating the ZP and fusing to the oocyte membrane.

Animal experiments demonstrated that under in vivo conditions, few spermatozoa reach the ampulla; this indicates that the contribution of the ZP to polyspermy prevention is substantially lower than previously expected. Under in vivo conditions, the number of spermatozoa in the ampulla is regulated by oviduct movement, and, in most cases, a single spermatozoon penetrates the ZP [26]. More recently, it was shown that ZP-deficient mouse oocytes generated from transgenic technology could be fertilized by a single spermatozoon in the ampulla; this indicates that the ZP does not contribute to blocking polyspermy as previously thought.

4. Protection of the preimplantation embryo from immunological attack

Even if the ZP is removed from the cleaved embryo, the embryo can develop into the blastocyst stage in vitro but not in vivo. Embryos transplanted into the oviduct of pseudo-pregnant mice do not pass through the oviduct or reach the uterus [27]; the embryos are probably absorbed onto the surface of the oviduct epithelium. However, embryos at the blastocyst stage can migrate in the oviducts and reach the uterus. Oviduct epithelial cells recognize early cleaved embryos as non-self antigens and eliminate them by immunological attack, whereas, blastocyst-stage embryos are recognized as self-antigens. Therefore, the ZP protects earlier stage preimplantation embryos from attack by the mother’s cells during oviduct transportation. More recently, it was proposed that the ZP or ZP derivatives could act as intrinsic signals to the maternal immune system for implantation susceptibility [28].

III) ZP-related Infertility

1. Antibodies against the ZP

Clinical studies have shown that anti-ZP antibodies are frequently associated with unexplained infertility [2–4]. Controversial results, stating that anti-ZP antibodies were not related to infertility, have also been published [29, 30]. Recently, there have been reports that showed an association between elevated anti-ZP antibody titers and poor fertilization rates in conventional in vitro fertilization (IVF). Some studies focused on follicular fluid rather than blood sera and documented a high detection frequency of anti-ZP antibodies in cases of unexplained infertility [31, 32]. The presence of anti-ZP antibodies in follicular fluid is a plausible cause of poor fertilization, because follicle fluid encloses the oocyte before ovulation. Some clinicians have recommended ICSI rather than conventional IVF, because repeated follicular puncture tends to increase the levels of circulating anti-ZP antibodies [33, 34]. Our animal experiments showed that immunization with solubilized pig ZP antigens produced histological features of empty follicles composed only of granulosa cells [35].

2. Oocyte-lacking follicles caused by ZP deficiency

The occurrence of oocyte-lacking follicles, empty follicle syndrome (EFS), is often observed in reproductive medicine. EFS is usually considered to be derived from inadequate stimulation or oocyte aspiration errors [36, 37]. However, as mentioned previously, GEFS might possibly arise from a genetic abnormality in ZP proteins. Indeed, animal experiments demonstrated that mice knocked out for the genes that encode zona proteins, ZP2 and ZP3 showed growth of follicles without oocytes similar to GEFS reported in humans [17, 18]. The mice were confirmed to be completely infertile [14, 15]. Anti-

Table 1. Antigens analyzed in contraceptive vaccine studies

Naturally occurring ZP glycoprotein
Deglycosylated ZP proteins
Recombinant ZP proteins
Synthetic peptides (B-cell epitopes)
Conjugation with promiscuous T-cell epitope
Engineered multiple epitopes
Self-proliferating DNA vaccine (delivery system)

ZP autoantibodies can also induce empty follicles in the ovary [35]. These follicles could potentially develop into GEFS during maturation.

IV) ZP-based Immunocontraceptive Vaccines

In the 1970s, anti-ZP antibodies were reported to inhibit fertilization and block sperm binding to the ZP [38–40]. Since then, several studies have revealed that active immunization with pig ZP components, which contain a common antigen (s) in various mammalian animals, produced anti-ZP antibodies that interfere with fertility, including in nonhuman primates [41–43]. By application of this immunization, intensive studies have been conducted to develop immunocontraceptive vaccines. The progress of ZP-based contraceptive vaccine studies is summarized in Table 1; the studies however revealed a conflict between achievement of sufficient efficacy and adverse side effects. Early studies showed that infertility was induced in immunized animals, but the effect was mainly a result of impairment of ovarian function rather than fertilization blocking. When using naturally occurring ZP materials as an immunogen, antibodies raised against contaminated somatic cell components were thought to impair ovarian functions.

To exclude undesired impairment of ovarian function, deglycosylated ZP proteins were utilized, because the carbohydrate moieties in the ZP glycoproteins were thought to be cross-reactive with somatic tissues [43]. However, these trials failed to prevent ovarian impairment as a contraceptive effect. Subsequently, recombinant ZP proteins that were not contaminated by somatic cells were examined to determine whether contraceptive effects can be generated without side effects [44–47]. The antigen preparations elicited a contraceptive effect concomitantly with ovarian failure, although they included neither somatic antigens nor carbohydrate antigens.

Subsequently, studies have focused on a sequential small peptide epitope that inhibits fertilization without any adverse side effects (Table 1). Millar *et al.* demonstrated that a synthetic peptide of a small region of ZP3 composed of 16 amino acids could produce a fertilization-blocking antibody without adverse side effects [48]. Such a small peptide that produces a specific antibody is called a B-cell epitope, because it stimulates B lymphocytes reactive to the small peptides. Several small peptides were identified as T-cell epitopes that induce cytotoxic T-cells which cause ovarian pathology. These results were encouraging for distinguishing B-cell epitope peptides that induce desired fertilization-blocking antibody and T-cell epitope peptides that induce undesired ovarian pathology from within ZP proteins. Based on this idea, several studies have attempted to identify fertilization-blocking B-cell epitopes that do not induce cytotoxic T-cell activity [48–50].

Numerous monoclonal antibodies against pig ZP proteins were produced to identify candidates for B-cell epitopes. We also generated monoclonal antibodies to block human sperm from binding to the human ZP [51, 52]; among them, a monoclonal antibody recognized the amino-terminal region of ZP2 and showed strong inhibition of human sperm binding to the human ZP. The several peptides that corresponded to the region of each animal were synthesized and conjugated with carrier protein in model experiments. The antibodies were successfully produced by homologous immunizations in rabbits, hamsters, and dogs. The raised antibodies inhibited sperm binding to the ZP in vitro, but ovarian pathology was not avoidable [53, 54]. As alternative strategies, conjugation of B-cell epitopes with promiscuous T-cell epitopes and engineering of multiple epitope vaccines have been attempted (Table 1). As a vaccine delivery system, self-proliferating DNA that encodes ZP peptides are attractive, because immunogens are automatically produced from introduced DNA in animals and immunostimulation happens consistently without booster injections [55–58]. The desired efficacy and undesired side effects should be carefully investigated before application. At this moment, successful immunocontraceptive vaccines are limited to wildlife, such as in populations of feral horses, white-tailed deer, African elephants, gray seals, and marsupials. In these trials, the native pig ZP was used as an immunogen.

Conclusion

The mammalian ZP has been studied by biologists and medical researchers since the 1970s. In many animal species, the molecular interactions of the ZP and sperm still remain to be determined. Recently, scientific evidence produced by advanced technologies such as live-cell imaging, single-cell analysis, and production of

multiple gene-modified animals seems to have further confused this issue. Natural events may be far more complicated than expected. From a clinical perspective, numerous researchers have shown that autoantibodies that are reactive to self-ZP antigens cause infertility because of interference with sperm binding of the ZP. However, opposing ideas have also been proposed. Although a significant correlation between anti-ZP antibody and infertility has not yet been established, modern assisted reproductive technologies, such as ICSI, provide an effective strategy for treating infertility. ZP-based contraceptive vaccines for human use have also been studied for several decades, but they are still a long way from achieving satisfactory efficacy and safety.

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