MINIREVIEW

The Zona Pellucida Genes Encode Essential Proteins for Mammalian Fertilization and Early Embryogenesis (43171C)

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In mammals, development begins at the time of fertilization when a mature sperm fuses with an ovulated egg to form a one-cell zygote. Each gamete contains specific macromolecules that mediate the carefully orchestrated events of fertilization. These molecules must ensure the proper approach and recognition of sperm and egg as well as their subsequent fusion. Following fertilization there must be mechanisms to prevent polyspermy and to protect the embryo during preimplantation development. Over the past decade there has been an explosive growth in understanding some of the macromolecules involved in mammalian fertilization (1, 2). Because much of this information has been ascertained in the mouse, it will serve as the focus of this review.

Oogenesis in the mouse results in the formation of a 70 μm in diameter oocyte surrounded by an 7-μm thick extracellular matrix, the zona pellucida. During ovulation, the egg completes the first meiotic division and begins its passage through the oviduct. The mature epididymal sperm is approximately 120-μm long and consists of a trail, a mid-piece, and a head. The sperm acrosome lies on the anterior portion of the head and contains a mixture of lytic enzymes, including acrosin. The epididymal sperm ejaculated during coitus undergo capacitation (a poorly characterized process that results in increased sperm membrane fluidity) in the female reproductive tract. It appears that fewer than 100 sperm successfully arrive in the oviduct where only a few penetrate the outer investments (cummulus oophorus) of the ovulated egg. Following an initial loose attachment of the sperm to the zona, a tight binding triggers the sperm acrosome reaction, thereby releasing lytic enzymes that may be important for the penetration of the sperm through the zona pellucida. The sperm then enters the perivitelline space between the zona and the egg's plasma membrane and the membranes of the two gametes fuse. The entire sperm enters the egg's cytoplasm where the sperm nuclear DNA undergoes decondensation and is repackaged on oocyte histones into the male pronucleus. At the time of fusion with the sperm, the egg completes the second meitotic division and the remaining haploid DNA is formed into the female pronucleus. The DNA in the one-cell zygote is replicated and the first cell division results in a two-cell embryo.

The zona pellucida is a structure that is crucial to fertilization and early development. It is synthesized by oocytes and secreted to form an extracellular matrix that surrounds the growing oocyte, the ovulated egg, and the preimplantation embryo. The mouse zona pellucida is composed of three sulfated glycoproteins designated ZP1, ZP2, and ZP3. The zona mediates initial sperm-egg interactions; it is responsible for induction of the sperm acrosome reaction. After fertilization, it serves as a major block to polyspermy and it facilitates the passage of the preimplantation embryo down the oviduct (3). Recent investigations into the molecular genetics of the zona pellucida have provided detailed information of the structure of the zona genes and their developmental expression during oogenesis. These genes not only encode proteins that play a vital role in fertilization and early development but their expression serves as a marker of oocyte growth and differentiation.

Structure of the Mouse Zona Pellucida Genes

Zp-2 and *Zp-3* are present in the mouse genome as single copy genes. *Zp-2* is composed of 18 exons

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ranging in size from 45 to 190 bp spanning 12.1 kb of DNA on mouse chromosome 7, 11 cm distal to *Tyr*. *Zp*-3 contains eight exons ranging in size from 92 to 338 bp extending over 8.6 kb of DNA on mouse chromosome 5, 9.2 cm distal to *Gus* (Fig. 1) (4–7).

The nucleic acid sequence of full-length cDNA of the mouse genes has been used to deduce the structure of the two zona transcripts and the resultant proteins (4, 8). Both ZP2 and ZP3 mRNAs have short 5′ (30 and 29 nt, respectively) and 3′ (32 and 16 nt, respectively) untranslated regions. Although not unheard of, these motifs are unusual and may play a role in the stability of the zona transcripts during oogenesis. The 2.4-kb polyadenylated ZP2 mRNA encodes a 713-amino acid protein that contains a 34-amino acid signal peptide to direct its secretion. The protein contains seven potential *N*-linked glycosylation sites, six of which are derivatized and more than 100 potential *O*-linked glycosylated sites. The 1.5-kb polyadenylated ZP3 mRNA codes for a 424-amino acid polypeptide chain that contains a 22-amino acid signal peptide, 6 potential *N*-linked glycosylated sites (3–4 of which are derivatized) and more than 70 potential *O*-linked glycosylation sites. The sequences of the two mRNA and their resultant proteins are unrelated to sequences found in current data bases and there is nothing to suggest that the two genes have a common ancestry. However, there are two five-amino acid sequences identities between the two zona proteins, one of which (Val-Ser-Leu-Pro-Gln) is located at the amino terminus of the secreted ZP2 proteins and at the carboxyl terminus of the ZP3 protein. In addition, both proteins have a very hydrophobic region at their carboxyl terminus. These protein domains may serve as signals for the intracellular trafficking of the zona proteins or may be important for the interaction of the two zona proteins with each other or with ZP1 to form filaments in the extracellular zona pellucida (see below).

Developmental Expression of the Zona Pellucida Genes

The mouse ovary at birth contains many thousands of resting oocytes (13 μm). These oocytes are in the prophase of first meiotic division and are surrounded by a single layer of spindle-shaped granulosa cells. By unknown mechanisms, a cohort of these oocytes enter into a 2-week growth phase during which their diameter increases to 70 μm. This growth phase is characterized by the synthesis and accumulation of macromolecules, proliferation of the surrounding granulosa cells, and the laying down of the extracellular zona pellucida. After the growth phase, the granulosa cells continue to multiply over several additional days to form a 600-μm follicle. The encased oocyte then undergoes meiotic division.

The appearance of the zona pellucida serves as a unique marker of oocyte growth and development. It is anticipated that, by investigating the molecular mechanisms that control the expression of the genes encoding the zona pellucida proteins, we will better understand signals that are responsible for the growth and differentiation of the female germ cells. Both *Zp-2* and *Zp-3* are expressed uniquely in ovarian tissue where their expression can be further localized to oocytes. Neither transcript is detected in the surrounding granulosa cells (Fig. 2). The zona genes are not expressed in resting oocytes but both ZP2 and ZP3 transcripts rapidly accumulate as the oocytes begin to grow and in 50 μm in diameter oocytes they represent 1% and 0.4% of the poly(A)$^+$ mRNA, respectively. In the latter stages of oocyte growth, their abundance declines dramatically and in ovulated eggs, it is less than 5% of the peak levels (4, 9, 10).

The rate of zona protein synthesis closely follows the abundance of the zona transcripts during oogenesis. Resting oocytes do not have a zona pellucida and zona protein synthesis is first observed in growing oocytes. In the later stages of oocyte growth, the rate of zona protein biosynthesis decreases and there is no detectable

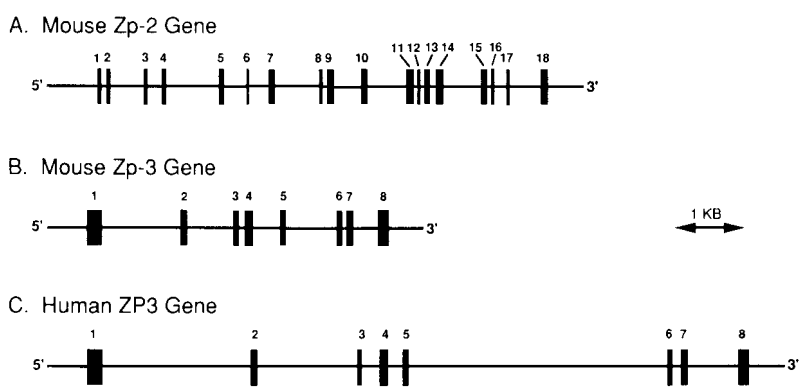

Figure 1. Exon map of mouse zona pellucida genes. (A) Mouse *Zp-2* with 18 exons spanning 12.1 kbp. (B) Mouse *Zp-3* with eight exons spanning 8.6 kbp. (C) Human *ZP3* with eight exons spanning 18.3 kbp.

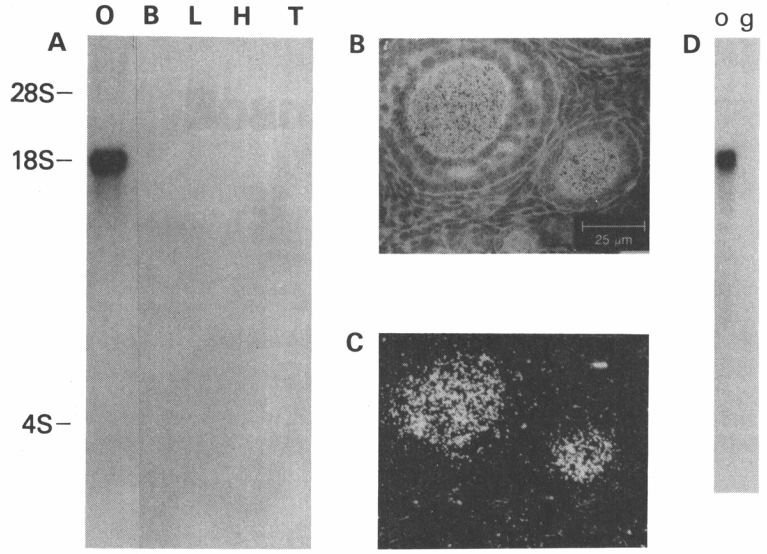

Figure 2. Oocyte-specific expression of mouse *Zp-2* and *Zp-3*. (A) Northern blot of RNA probed with ^{32}P-labeled ZP3 cDNA (O, ovary, B, brain; L, liver; H, heart; T, testis). (B) *In situ* hybridization of ovarian sections with ^{35}S-labeled antisense ZP3 transcripts. (C) Same as B viewed with darkfield optics. (D) Northern blot of RNA from oocytes (o) and granulosa cells (g) probed with ^{32}P-labeled ZP2 cDNA.

zona pellucida synthesis in ovulated eggs (11, 12). This cessation appears to be due both to the low levels of zona transcripts found in ovulated eggs and to their inability to be translated after deadenylation (as has been noted for other oocyte mRNA). The ZP2 and ZP3 proteins are synthesized as 80,217-Da and 46,307-Da precursor proteins, respectively. Each contains a signal peptide that is cleaved off resulting in a core ZP2 protein of 76,373 Da and core ZP3 protein of 43,943 Da (4, 8). The zona proteins are subsequently glycosylated, sulfated, and secreted as mature ZP2 and ZP3 of 120,000–140,000 Da and 83,000 Da, respectively (12, 13).

Little is known about the mechanisms by which the zona proteins are assembled into the insoluble extracellular matrix that surrounds growing oocytes. Electron microscopy indicates that the zona pellucida is comprised of long intertwining filaments made up of dimers of ZP2 and ZP3 that are cross-linked by ZP1 (a disulfide-linked dimeric protein) (14). The zona structure must be sufficiently flexible to accommodate the 150-fold increase in the volume of the growing mouse oocyte as it increases from 13 to 70 μm in diameter, yet sturdy enough to mediate fertilization and protect the growing embryo. Intact isolated zona can be dissolved by extremes of pH or by heat. However, when relieved of the perturbant, the zona proteins stay in solution. Presumably, the interaction of the zona proteins with one another makes them insoluble but how or where this assembling takes place remains to be determined.

Biologic Function of the Zona Pellucida

The zona pellucida encases the oocyte during growth and development, modulates the events of species-specific fertilization, and protects the preimplantation embryo. ZP3 acts as the initial sperm receptor via *O*-linked oligosaccharide side chains and the presence of its polypeptide backbone is important for the induction of the sperm acrosome reaction. ZP2 acts as a secondary sperm receptor and it, along with ZP3, is modified following fertilization and is presumed to play a role in the post fertilization block to polyspermy (3, 15).

Although the zona pellucida glycoproteins have been characterized in considerable detail, less is known about the corresponding ligands on the surface of the sperm (2). In the oviduct, the ovulated mouse egg is surrounded by a cummulus oophorus that contains cumulus cells and an extracellular matrix composed primarily of glycosaminoglycans. Only capacitated, acrosome-intact sperm appear to be able to pass through this investment and, in the mouse, only acrosome-intact sperm bind to the zona pellucida. Over the last decade a number of candidate sperm surface macromolecules have been proposed (proteinases, sugar transferases, sugar binding proteins, etc.). More recent investigations have suggested two alternative candidates: a 56,000-Da protein located on the surface of the intact acrosome and a 95,000-Da sperm surface protein that has tyrosine kinase activity (16, 17). It is postulated that aggregation of this latter protein in the sperm plasma membrane by ZP3 triggers a signal transuction cascade that results in the acrosome reaction (17, 18). This exocytotic event releases lytic enzymes that, when coupled to the forward motility of the sperm, leads to the penetration of the zona pellucida. It is likely that more than one interaction is responsible for the binding of sperm to the zona, both initially and during penetra-

tion. Some of the sperm ligands may become available for binding only after the sperm acrosome reaction. Thus, macromolecules on the surface of the inner acrosomal membrane (proacrosin, sugar binding proteins, etc.) also appear to play an important role in sperm-egg interactions and successful penetration of the zona pellucida.

After fertilization, the zona is modified to prevent polyspermy. The preovulatory oocyte contains several thousands of cortical granules (0.2–0.8 μm) uniformly distributed around the cortex (19, 20). During meiotic maturation, about 25% of these granules are lost through exocytosis, leaving a cortical granule-free region at the oocyte metaphase II chromosome plate. This may cause local modification of the zona that precludes sperm penetration and, thus, orient the point of fertilization to the opposite hemisphere. At fertilization the remaining granules fuse with the egg's plasma membrane in the cortical granule reaction and release their contents (proteinase, glycosidases, etc.) into the perivitelline space. These enzymes modify the zona pellucida: ZP3 no longer has sperm receptor activity (15) and ZP2 undergoes a proteolytic cleavage associated with the block to polyspermy (21).

During the 3–4 days of preimplantation development, the zona pellucida continues to surround the embryo and prevents interactions with oviductal epithelial cells that could hinder passage and cause ectopic development. With each division, the size of individual daughter cells decreases as the volume of the total cytoplasm remains fairly constant. After entry into the uterine cavity, the blastocyst hatches out of the zona pellucida and then is free to implant by interacting with the cells of the uterine wall. Although mechanical forces exerted by the growing embryo may play a major role in hatching, there is also evidence that specific enzymes located in the mural trophectoderm may weaken the zona and permit the embryo to escape (22).

Conservation of the Zona Genes among Mammals

The *Zp-2* and *Zp-3* genes are conserved among mammals (mouse, rat, rabbit, dog, pig, cow, and human) and the degree of conservation of *Zp-3* varies with pig and rabbit being less related to mouse than rat, dog, cow, and human zona genes (10). The human *Zp-3* gene has been isolated recently and contains eight exons that are virtually identical in size to those of the mouse *Zp-3* gene, although the overall size of the genetic locus (18.3 kb from Exons 1–8) is about twice as large (23). Using the polymerase chain reaction, a full-length cDNA of human ZP3 was isolated from human ovarian poly(A)$^+$ RNA and used to deduce the structure of human ZP3 mRNA. The human ZP3 transcript is remarkably similar to the mouse ZP3 mRNA. Both have short 5′ and 3′ untranslated regions and both have a single open reading frame of 1272 nt that encodes a 424-amino acid protein. The deduced amino acid sequences of the human and mouse ZP3 protein are 67% identical and, as evidenced by similarity of their predicted secondary structure, many substitutions are conservative (Fig. 3). The size of three other ZP3 transcripts (mouse, rat, and rabbit) are indistinguishable one from another, suggesting that the overall structure ZP3 mRNA is conserved among mammals (8).

The similarities of the ZP3 proteins among species may be important in providing structural integrity to the extracellular zona pellucida and in positioning protein and carbohydrate moieties so that they can participate in sperm-egg interactions. If, as is the case in the mouse, human ZP3 functions as the sperm receptor *in vivo*, the regions of maximal differences may play a role mediating high affinity interactions that lead to fertilization for each species.

Future Directions

Oocyte-Specific Gene Expression. The mechanisms that control the onset of coordinate expression of the zona pellucida genes is under intense investigation. The promoter regions of the mouse and human *Zp-2* and *Zp-3* genes have been compared one with another and DNA elements ranging in size from 8 to 12 bp in size have been found at comparable distances from the start of transcription in all four genes. Promoters containing mutations in each of these elements can be coupled to reporter genes and the functional significance of each element assayed by microinjection of the construct into growing oocytes. The identification of proteins that bind to DNA sequences with function will provide information on the mechanisms that regulate the expression of the zona pellucida genes. In addition, by investigating the molecular biology of these trans-acting factors, greater insight into the signals that induce oocyte growth and differentiation may be gained.

Biologic Function of the Zona Pellucida. The cloning of the zona genes, each in a species for which *in vitro* fertilization is possible, may provide the necessary reagents with which to gain additional knowledge of those protein and carbohydrate domains that are critical for species-specific fertilization. If, as suggested by the data for mouse and human ZP3, the structures of the zona proteins are similar, mouse-human chimeric proteins with particular regions of the mouse polypeptide chain substituted with human sequence can be tested for biologic activity. These reagents may be particularly useful in defining sperm surface macromolecules important for sperm-zona interactions. In addition, transgenic mouse lines expressing human (or other species) zona pellucida genes can be established. If the protein product of the transgene participates in

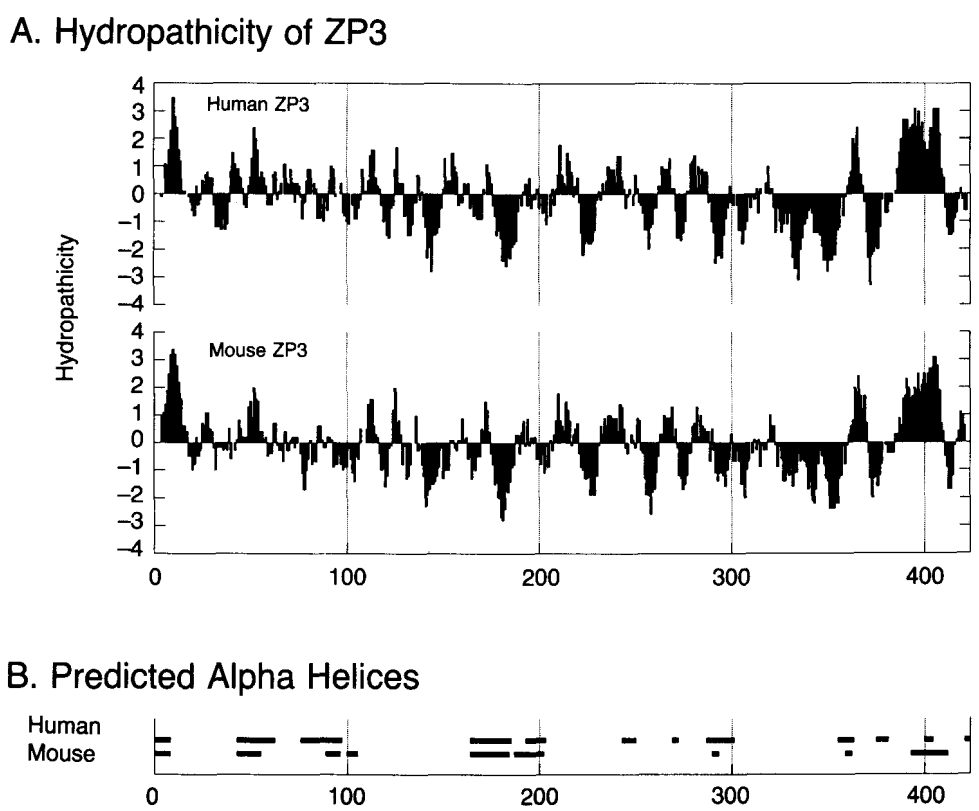

Figure 3. Secondary structure of mouse and human ZP3 protein. (A) Hydropathicity plot of mouse and human ZP3 proteins indicating degree of conservation between the two. (B) Distribution of potential α-helical structure of each ZP3 protein (from ref. 23).

the zona pellucida, these animals may be useful laboratory reagents for assaying sperm function in *in vitro* fertilization clinics.

Zona Pellucida-Based Immunocontraception. The mammalian zona pellucida has been recognized as a candidate target for a contraceptive vaccine since the early 1970s. However, vaccination with purified zona pellucida components that induce antizona antibodies has resulted in widespread ovarian histopathology and abnormal ovarian function. Recently, immunization with a synthetic ZP3 peptide (amino acids 328–343) coupled to a carrier protein has been shown to result in long-term, reversible contraception (24). The zona peptide contains a 7-amino acid epitope that elicits antizona pellucida antibodies and contains a partially overlapping peptide that induces ovarian histopathology in some, but not all, inbred strains of mice (Rhim et al., submitted for publication). If these two effects can be separated, contraception based on the zona pellucida may be feasible. Because the genes that code for zona pellucida proteins are conserved among mammmals, this strategy, that involves vaccination with self-zona pellucida peptides, may be applicable to other mammalian species.

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