

# The zona pellucida: using molecular genetics to study the mammalian egg coat

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An extracellular matrix that mediates critical steps in fertilization and early development surrounds all vertebrate eggs. In mice and humans, this matrix is known as the zona pellucida and comprises three glycoproteins: ZP1, ZP2 and ZP3. Homologues of these proteins isolated from other vertebrates have conserved protein motifs that may be important for establishing a common fibrillar structure. However, specific but contradictory biological roles have been assigned to individual egg coat proteins based on assays *in vitro* in a wide range of species. Mouse lines lacking either ZP1 or ZP3 have been established with abnormal or absent zona matrices and varying degrees of infertility to examine zona structure and function *in vivo*. By crossing mouse lines lacking individual zona proteins with those expressing human homologues, the structural integrity of the zona matrix can be restored. Because mouse and human spermatozoa exhibit order-specific binding to the zona pellucida, mice with 'humanized' chimaeric zonae may provide an experimental system to elucidate the molecular basis of sperm–zona interaction.

All vertebrate eggs are surrounded by an extracellular matrix that is variously referred to as the chorion (fish), the vitelline envelope (frogs), the perivitelline membrane (birds) and the zona pellucida (mammals). Despite differences in terminology, these extracellular egg matrices perform similar functions during fertilization and early embryonic development and have remarkably similar ultrastructures: all are fibrous matrices with conserved individual components featuring common protein domains. These motifs have been preserved over millions of years of evolution, from fish to mammals, and are presumed to serve important structural or biological functions.

However, the molecular interactions that occur during fertilization remain specific to different vertebrates, and it is clear, particularly in mammals, that the extracellular egg matrix provides an order-specific barrier to fertilization at the level of sperm–egg binding (Yanagimachi, 1994). Studies into the nature of these extracellular egg matrices revolve around determining the significance of the conserved structural motifs and how the differences between the matrix proteins result in order-specific binding of spermatozoon to egg. The mouse is an attractive experimental model for these investigations because its genome can be manipulated to create novel phenotypes that illuminate the roles of the individual matrix components in determining the structure and function of the extracellular zona pellucida.

## Composition of the mouse and human zonae pellucidae

The mouse zona pellucida is composed of three sulfated glycoproteins, each designated for its mobility during SDS-PAGE, in which mouse ZP1, ZP2 and ZP3 have apparent molecular masses of 180–200, 120–140 and 83 kDa, respectively (Bleil and Wasserman, 1980a; Shimizu *et al.*, 1983). ZP1 forms a dimer in the zona matrix with 120 kDa monomeric subunits. Sequencing

of cDNAs encoding the zona proteins has enabled the determination of the primary structure of ZP1 (623 amino acids), ZP2 (713 amino acids) and ZP3 (424 amino acids) (Ringuette *et al.*, 1988; Liang *et al.*, 1990; Epifano *et al.*, 1995). The predicted molecular masses of the protein cores of ZP1 (68 kDa), ZP2 (80 kDa) and ZP3 (46 kDa) are about half the apparent molecular mass of each zona protein indicating significant post-translational modification. Much of this appears to be glycosylation that would permit the matrix to maintain a high state of hydration and may be important for sperm penetration through the zona pellucida. In addition, assays *in vitro* have implicated some of the carbohydrate residues in mediating sperm–zona interactions in mice (Florman and Wasserman, 1985; Bleil *et al.*, 1988; Miller *et al.*, 1992).

The nomenclature of human zona proteins was similarly based on the mobilities of ZP1 (90–110 kDa), ZP2 (64–78 kDa) and ZP3 (57–73 kDa) in SDS-PAGE (Shabanowitz and O'Rand, 1988). However, using antibodies specific to each zona protein (Gupta *et al.*, 1998) and on the basis of comparisons of primary structures deduced from cDNA clones, it is clear that the 64–78 kDa human protein corresponds to mouse ZP1 and the 90–110 kDa human protein corresponds to mouse ZP2. In this review, sequence-based homologues found in all vertebrates, including humans, is referenced according to homology to mouse ZP1, ZP2 or ZP3 (Table 1) to minimize confusion. The length and the sequence identity of human ZP2 (715 amino acids; 61% identical to mouse) and ZP3 (424 amino acids; 67% identical to mouse) are quite high (Chamberlin and Dean, 1990; Liang and Dean, 1993). However, human ZP1 (540 amino acids) is shorter than mouse ZP1 (623 amino acids) and only 43% identical (Harris *et al.*, 1994; Epifano *et al.*, 1995). As part of the Human Genome Project, a gene potentially encoding a 638 amino acid protein that is 65% identical to mouse ZP1 has been

Table 1. Vertebrate egg coat proteins

|  | Egg coat protein I | Egg coat protein II | Egg coat protein III | Tissue origin           | Predicted TM domain |
|--|--------------------|---------------------|----------------------|-------------------------|---------------------|
| Mouse<br>( <i>Mus musculus</i> )                     | ZP1 <sup>a</sup>   | ZP2                 | ZP3                  | Oocytes                 | Yes                 |
| Human<br>( <i>Homo sapiens</i> )                     | ZP1                | ZP2                 | ZP3                  | Ovary                   | Yes                 |
| Rabbit<br>( <i>Oryctolagus cuniculus</i> )           | r55                | r75                 | r45                  | Granulosa cells/oocytes | Yes                 |
| Pig<br>( <i>Sus scrofa</i> )                         | ZPB                | ZPA                 | ZPC                  | Oocytes                 | Yes                 |
| Rat<br>( <i>Rattus norvegicus</i> )                  | ZP1                | ZP2                 | ZP3                  | Ovary                   | Yes                 |
| Bonnet monkey<br>( <i>Macaca radiata</i> )           | ZP1                |                     | ZP2                  | Oocytes                 | Yes                 |
| Brushtail possum<br>( <i>Trichosurus vulpecula</i> ) | ZPB                | ZP2                 | ZP3                  | Ovary                   | Yes                 |
| Chicken<br>( <i>Gallus gallus</i> )                  |                    |                     | ZP3/ZPC              | Granulosa cells         | Yes                 |
| Frog<br>( <i>Xenopus laevis</i> )                    |                    | gp69/ZP2/ ZPA       | gp43/ZP3/ ZPC        | Oocytes                 | Yes                 |
| Carp<br>( <i>Cyprinus carpio</i> )                   |                    | ZP2 <sup>b</sup>    | ZP3                  | Oocytes                 | No                  |
| Medaka<br>( <i>Oryzias latipes</i> )                 | ChgH (Z1-1,2)      |                     | ChgL (ZI-3)          | Liver                   | No                  |
| Zebrafish<br>( <i>Danio rerio</i> )                  |                    | ZP2 <sup>b</sup>    | ZP3                  | Ovary                   | No                  |

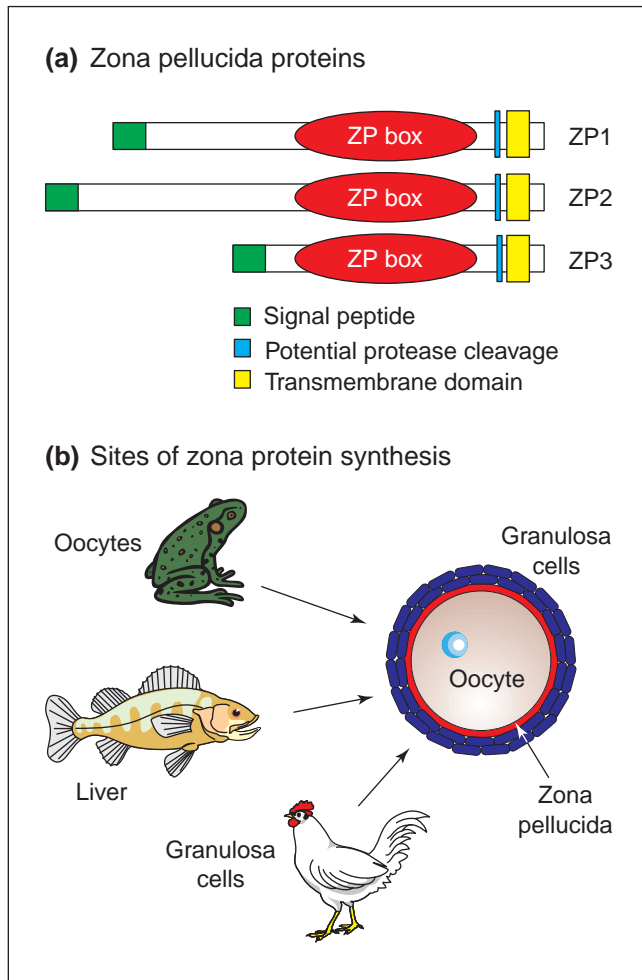
<sup>a</sup>References for primary structures: Ringuette *et al.*, 1988; Chamberlin and Dean, 1990; Liang *et al.*, 1990; Schwoebel *et al.*, 1991; Lee *et al.*, 1993; Liang and Dean, 1993; Yurewicz *et al.*, 1993; Harris *et al.*, 1994; Epifano *et al.*, 1995; Kolluri *et al.*, 1995; Murata *et al.*, 1995, 1997; Chang *et al.*, 1996, 1997; Gupta *et al.*, 1997; Kubo *et al.*, 1997; Yang and Hedrick, 1997; Akatsuka *et al.*, 1998; Mate and McCartney, 1998; Sugiyama *et al.*, 1998; Waclawek *et al.*, 1998; Haines *et al.*, 1999; Hughes and Barratt, 1999; McCartney and Mate, 1999; Tian *et al.*, 1999; Wang and Gong, 1999.

<sup>b</sup>These proteins share a similar degree of homology to mouse ZP1 and ZP2.  
TM, transmembrane domain.

reported, but whether it is expressed in oocytes and incorporated into the human zona pellucida remains to be determined experimentally (Hughes and Barratt, 1999).

The mouse and human zona proteins share certain protein motifs that are conserved in other vertebrates (Fig. 1a; Table 1). Each protein has a signal peptide that directs it into a secretory pathway and a transmembrane domain near its carboxyl terminus, upstream of which (20–30 amino acids) is a basic amino acid cleavage recognition sequence (absent in human ZP1) for furin, a protease located ubiquitously in the trans-Golgi. However, the most striking degree of conservation among zona proteins is the signature zona domain: a 260 amino acid motif defined by eight conserved cysteine residues that is present in several extracellular proteins, some of which participate in fibrillar matrices (Bork and Sander, 1992; Legan *et al.*, 1997). In addition, a potential trefoil domain (six conserved cysteine

residues linked 1–5, 2–4, 3–6) has been identified just upstream of the zona domain in ZP1 homologues (Sommer *et al.*, 1999). Although three-dimensional structures have not been determined for any of the zona proteins, a structural model has been proposed for the mouse zona pellucida on the basis of ultrastructural and biochemical analyses (Greve and Wasserman, 1985; Green, 1997). This model suggests that ZP2 and ZP3, which are present in the zona at roughly equimolar concentrations, form heterodimeric filaments and that these filaments are cross-linked by homodimers of ZP1, which is the least abundant zona protein in mice. How the three zona proteins traffick through the cell, presumably as soluble proteins, but form an insoluble matrix that surrounds the growing oocytes is unknown. The observation that a similar filamentous structure is formed by vertebrates in which the egg matrix proteins are synthesized in the liver and transported to the ovary



**Fig. 1.** Structural motifs and site of synthesis of the zona proteins. (a) Each of the three zona pellucida proteins (ZP1, ZP2 and ZP3) have conserved protein domains. The conserved 260 amino acid zona domain is termed the 'ZP box' and the other domains are indicated. (b) The site of synthesis of egg coat proteins has been reported in oocytes (in mammals, frogs and fish), liver (in fish) and granulosa cells (in chickens and mammals). Many, if not all, precursor forms undergo N-terminal and C-terminal processing before incorporation into the extracellular egg matrix variously known as the zona pellucida (in mammals), perivitelline envelope (in birds), vitelline envelope (in frogs) or chorion (in fish).

(Yamagami *et al.*, 1992) or in the granulosa cells (Waclawek *et al.*, 1998) indicates a complex regulatory mechanism to ensure correct zona pellucida formation (Table 1).

Although the zona proteins are sufficiently conserved to facilitate the construction of a fibrillar matrix, they must be sufficiently divergent to allow for order-specific, sperm binding features in their supramolecular zona structure. The morphology of spermatozoa varies considerably among vertebrates and among mammalian orders. Human spermatozoa (60  $\mu\text{m}$  in length), which are half as long as mouse spermatozoa (125  $\mu\text{m}$ ) must penetrate a zona matrix (15  $\mu\text{m}$ ) that is twice as thick as that of the mouse (7  $\mu\text{m}$ ). The heads of human spermatozoa have rounded edges and appear quite flat, whereas mouse

sperm heads are pointed with a characteristic hook. Thus, it would seem advantageous to these two mammals to vary the supramolecular structure of the zona matrix to enhance the binding of their cognate spermatozoa, which are so morphologically distinct. The markedly different amounts of carbohydrate side chains associated with the zona proteins from different orders could provide additional variables to promote the specificity of sperm binding. Alternatively, alterations could be imposed on the matrix by differences in the less well conserved regions of the polypeptide backbone of individual zona proteins.

### The egg envelopes of other vertebrates

The conserved zona domain has been observed in egg coat proteins isolated from many species (Table 1) despite differences in their site of synthesis (Fig. 1b). In some teleost fish (for example, winter flounder and medaka), the egg envelope precursors are synthesized in the liver under the influence of oestrogens and are carried in the bloodstream to the ovary, where they are deposited in the vitelline layer of the developing eggs (Yamagami *et al.*, 1992). However, in other fish species (for example, carp, goldfish and sea bream), the egg matrix proteins are synthesized in growing oocytes (Chang *et al.*, 1996, 1997). In chickens, it appears that the egg envelope proteins are synthesized by the surrounding granulosa cells and deposited in the perivitelline membrane of the egg (Waclawek *et al.*, 1998; Takeuchi *et al.*, 1999) but, in *Xenopus* and mice, growing oocytes synthesize and secrete the zona proteins to form the extracellular matrix. In *Xenopus* and mice, C-terminal processing of the zona proteins may release them from the membrane (where they are 'tethered' by their transmembrane domain) such that they can interact with each other to form the egg shell. Whether this release occurs during intracellular trafficking through the endoplasmic reticulum and Golgi or on the plasma surface is unknown. This processing may involve a conserved furin cleavage site (Yurewicz *et al.*, 1993) as has been reported for mouse ZP2 and ZP3 (Litscher *et al.*, 1999). In *Oryzias latipes*, a fish species that synthesizes its egg envelope precursors in the liver, these precursors that have no transmembrane domain also undergo C-terminal processing before incorporation into the vitelline envelope (Sugiyama *et al.*, 1999). This cleavage seems to occur at dibasic amino acid sites present in both fish and mammals. These findings indicate that this C-terminal processing is not only important for release of the zona proteins from the cellular membrane when they are produced either by oocytes or granulosa cells, but that it is also important for the ability of the secreted zona proteins to associate and form a fibrillar matrix.

### Functions of the zona pellucida

The zona pellucida has multiple functions during fertilization and preimplantation development. It mediates initial sperm-egg recognition and induces the acrosome reaction in spermatozoa, allowing for the continued penetration of spermatozoa through the zona and its subsequent fusion with the egg oolemma. Immediately after fertilization, the zona is modified by the contents of the cortical granules, released upon egg activation, such that spermatozoa can no longer bind to the matrix

and those already bound can no longer penetrate it. This process has been referred to as zona hardening and provides the major block to polyspermy in mammals. The zona matrix surrounds the developing embryo until the blastocyst stage, at which time it escapes from the zona and implants in the uterine wall.

How the zona pellucida accomplishes these tasks continues to be investigated, and results obtained in different vertebrates are contradictory as to the roles of the individual zona glycoproteins. Initial studies were shaped by the striking observation that SDS-PAGE-purified ZP3 (but not ZP1 or ZP2) inhibits sperm binding to ovulated mouse eggs *in vitro* in a dose-dependent manner (Bleil and Wasserman, 1980b). These studies were further refined to ascribe sperm binding activity to a class of O-linked oligosaccharide side chains (Florman and Wasserman, 1985) variously described as terminating in  $\alpha$ 1,3-galactose (Bleil and Wasserman, 1988) or N-acetylglucosamine (Miller *et al.*, 1992). However, genetic experiments *in vivo* have thrown the nature of the sugar moiety responsible for sperm-binding into question. Genetically engineered female mice that lack the galactosyl transferase required for the addition of  $\alpha$ 1,3-galactose are fertile (Thall *et al.*, 1995), as are male mice lacking the galactosyl transferase isoform thought to bind the terminal N-acetylglucosamine residues of ZP3 (Asano *et al.*, 1997; Lu and Shur, 1997). Although these genetic results indicate that sperm binding is not solely dependent on the aforementioned sugar determinants, either or both may participate in sperm–zona pellucida interactions along with other, yet to be ascertained, protein or carbohydrate domains.

The three mouse proteins (ZP1, ZP2 and ZP3) are 39–48% similar to three *Xenopus* proteins (gp37, gp69/64 and gp43/41) and are sufficiently conserved that individual mouse proteins expressed in frog eggs are incorporated into the surrounding vitelline envelope (Doren *et al.*, 1999). The *Xenopus* homologue of ZP2 (gp69/64), but not those of ZP1 (gp37) or ZP3 (gp43/41), has been shown to inhibit sperm binding to frog eggs (Tian *et al.*, 1997). The sperm binding activity is ascribed to a 27 amino acid N-terminal peptide and may involve an O-linked glycan (Tian *et al.*, 1999). Earlier data in mice also suggested a role for ZP2 in sperm binding, but as a secondary receptor that functions only after the initial interaction with ZP3 (Bleil *et al.*, 1988). In other vertebrates, ZP1 has been implicated in sperm binding. Recombinant rabbit ZP1 (r55) binds to the anterior surface of the sperm acrosome, and antibodies to the protein inhibit sperm binding (Prasad *et al.*, 1996). When expressed as recombinant protein, rabbit sp17, a sperm protein implicated in zona binding, can bind to either rabbit ZP1 (r55) or ZP3 (r45), raising the possibility of more than one binding molecule within the zona matrix (Yamasaki *et al.*, 1995). Earlier studies implicated the pig ZP1 homologue in sperm binding (Sacco *et al.*, 1989; Yonezawa *et al.*, 1995), but this binding activity is now attributed to the formation of ZP1–ZP3 heteroduplexes, which bind to pig sperm membranes *in vitro* (Yurewicz *et al.*, 1998). Thus, there is experimental evidence that homologues of each of the three major zona pellucida proteins mediate the binding of sperm to the vertebrate egg coat in at least one species. Whether sperm binding involves carbohydrate or protein domains remains an area of investigation.

After fertilization, the zona pellucida is altered by the contents of the cortical granules that are discharged into the

perivitelline space, and these modifications in the zona provide the major block to polyspermy. The only biochemical alteration correlated directly with this physiological change is the proteolytic cleavage of ZP2 near its N-terminus that has been observed in mice (Bleil *et al.*, 1981), pigs (Hasegawa *et al.*, 1994) and *Xenopus* (Tian *et al.*, 1999). Despite the cleavage of ZP2 upon fertilization, the N-terminal fragment of the protein remains bound to the parental ZP2 by disulphide bonds, and it is unclear how such a modification relates to the marked structural changes that preclude sperm binding and penetration. In fish, chorion hardening is associated with N-terminal cross-links of a Pro-X-Y repeat motif present in ZP1/2 fish homologues (Lee *et al.*, 1994). There also may be changes in ZP1 and ZP3 associated with the block to polyspermy, and cortical granule N-acetylglucosaminidase has been implicated in modifying mouse ZP3 (Miller *et al.*, 1993).

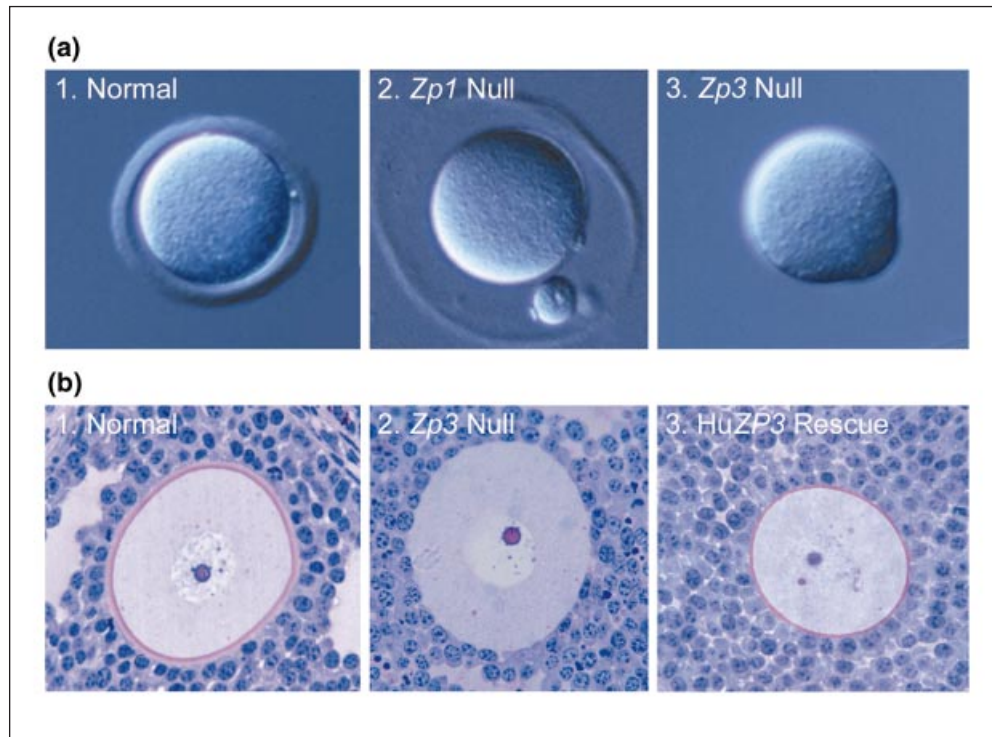
The zona continues to encase the fertilized egg during preimplantation development. This process affords protection for the early embryo during its transit down the mammalian oviduct. If zonae are removed before the four-cell stage and the embryos are transferred back into the oviduct, none implant on the uterine wall and complete gestation (Bronson and McLaren, 1970; Modlinski, 1970). Instead, the zona-free embryos adhere to the oviductal epithelium, fail to undergo continued cleavage, and disappear within 24 h. This critical role of the zona in preimplantation development has become even more apparent with the creation of genetically altered mice in which the zonae are either structurally abnormal or absent.

### Phenotypes of *Zp1*- and *Zp3*-null mice

Although the above observations make it clear that the zona pellucida plays a critical role in mammalian fertilization and early embryogenesis, the role of individual proteins or their carbohydrate sidechains in these processes has yet to be resolved satisfactorily. Evolutionarily, eutherian mammals are separated by < 130 million years (Kumar and Hedges, 1998), and individual zona proteins are well conserved among them. Mammals fertilize internally and speciation is adequately maintained by pre-mating (habitat, mate discrimination and physiognomy) and post-mating (hybrid lethality and sterility) determinants (O'Rand, 1988). Thus, despite the lack of concordance in the current literature, it seems unlikely that different classes of eutherian mammals would use different zona proteins for sperm binding.

In an attempt to complement *in vitro* studies, investigators have taken advantage of advances in transgenesis to specifically alter the genome of the mouse and create novel phenotypes. Embryonic stem cell technology has been used to establish mouse lines with null mutations in each of the three mouse zona proteins. Mouse oocytes lacking one of the three proteins continue to synthesize and secrete the other two proteins; however, the resulting mice exhibit structural defects in their zonae pellucidae that are associated with varying degrees of abnormal folliculogenesis and infertility.

Female mice with a *Zp1*-null mutation maintain a zona pellucida around their growing oocytes and ovulated eggs (Fig. 2a). However, the zona matrix is somewhat thinner than normal and is structurally compromised. In approximately 10% of growing follicles, the abnormal zona in the *Zp1*-null mice



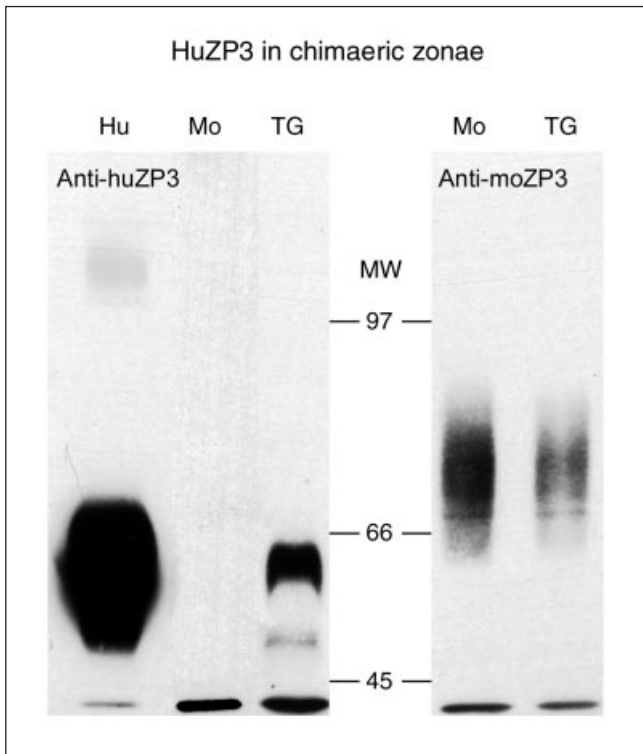
**Fig. 2.** Eggs and oocytes from genetically altered mouse lines. (a) Ovulated eggs isolated from normal, *Zp1*-null, and *Zp3*-null females. The zona matrix is significantly altered in *Zp1*-null mice and completely absent in *Zp3*-null females. (b) Histological sections of periodic acid Schiff (PAS)-stained ovarian tissue from normal, *Zp3*-null and huZP3 rescue mice. Oocytes in the huZP3 rescue animals were able to form a zona pellucida matrix, whereas *Zp3*-null oocytes were not.

results in ectopic localization of granulosa cells between the zona and the egg membrane. The structural impairment of the zona becomes more pronounced as follicles enter the preovulatory stage under the influence of gonadotrophins. The defective matrix results in an accentuated perivitelline space, often containing cumulus cells that have undergone the hormone-induced mucification reaction. Even though the *Zp1*-null females ovulate a similar number of eggs to normal females, their litter sizes are only half those of normal mice. This reduced litter size appears to be the result of a substantial loss of early embryos in the *Zp1*-null animals, most likely due to the inability of the compromised matrix to protect these early embryos adequately within the oviduct (Rankin *et al.*, 1999).

Mouse lines in which *Zp3* has been inactivated by targeted mutagenesis have also been established. In contrast to the relatively mild phenotype observed in *Zp1*-null females, mice lacking ZP3 have a striking absence of a zona pellucida (Fig. 2a,b). Thus, even though both ZP1 and ZP2 are being synthesized, they are inadequate to form a zona pellucida matrix, whereas ZP2 and ZP3 appear to be sufficient. The absence of a zona matrix in the *Zp3*-null mice impairs the ability of the ovary to form a well organized cumulus–oocyte complex in the preovulatory follicle. In *Zp3*-null mice, the egg is associated only loosely with the surrounding cumulus cells, and this probably prevents most of the ovulated, zona-free eggs from entering the infundibulum of the oviduct. After hormone stimulation, very few ovulated eggs are recovered in the oviduct and no two-cell

embryos are observed after mating with males proven to be fertile (Liu *et al.*, 1996; Rankin *et al.*, 1996). Thus, while the structurally impaired zona in the *Zp1*-null mice results in decreased fecundity, the absence of a zona in the *Zp3*-null results in sterility.

These data demonstrate that zona matrix formation absolutely requires ZP3, but not ZP1, and indicates that interaction between ZP3 and ZP2 initiates extracellular matrix formation. It also appears that the synthesis of each of the zona proteins occurs independently of the others, despite the coordinated transcription of the zona genes during folliculogenesis (Epifano *et al.*, 1995). The zona proteins synthesized in the absence of a zona matrix are still secreted, but are probably degraded in the extracellular space. A zona matrix can be created with only ZP2 and ZP3; however, the resulting matrix suffers from severe structural defects, resulting in abnormal folliculogenesis and decreased fecundity. Notwithstanding these defects, the ZP2–ZP3 matrix is sufficient for normal fertilization to occur, indicating that ZP1 is dispensable during sperm–egg interaction, induction of the acrosome reaction and for establishing a block to polyspermy. The phenotype of the *Zp1*-null female is consistent with an extant model of the zona structure (Greve and Wasserman, 1985; Green, 1997) in which ZP1 provides structural support for the matrix and does not participate directly in sperm interaction. Whether this finding is true in other species in which ZP1 has been implicated as the sperm receptor remains to be determined.

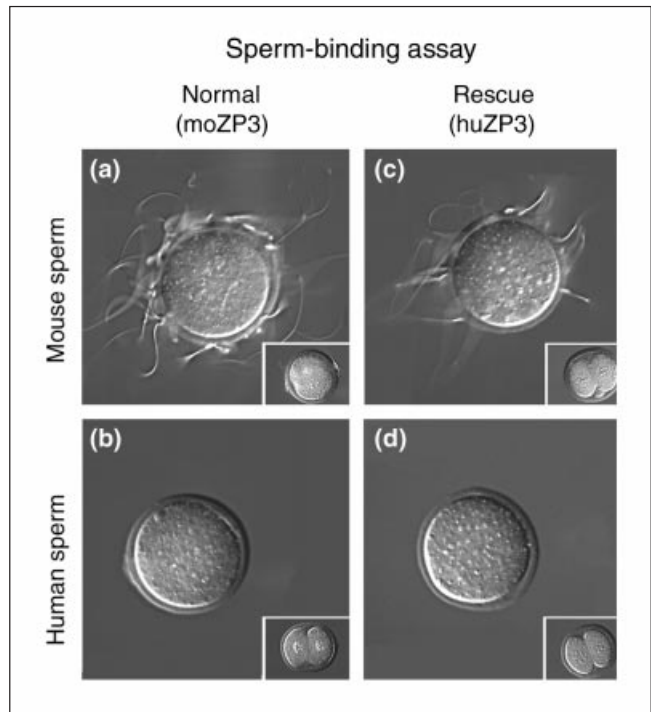


**Fig. 3.** Structure and function of human ZP3 rescue eggs. Western blot of zonae pellucidae from normal (Mo) and huZP3 transgenic (TG) eggs stained with monoclonal antibodies specific to human ZP3 (left) or mouse ZP3 (right). Human zonae pellucidae (Hu) were used as a control. HuZP3 expressed in the transgenic mice had a molecular mass indistinguishable from that of native human ZP3 (64 kDa) and distinct from endogenous mouse ZP3 (83 kDa).

### Biological function of ‘humanized’ zonae

The absence of a zona pellucida matrix in *Zp3*-null mice precludes an examination of the function of ZP3 in sperm–zona interaction. Therefore, an alternative genetic strategy has been devised that takes advantage of the observation that human spermatozoa will not bind to mouse zona pellucida (Bedford, 1977). Pronuclear injection has been used to establish mouse lines that express human ZP3 in growing oocytes. As judged by its mobility on SDS-PAGE, the human protein expressed in the mouse oocyte is modified after translation to the extent observed in native human ZP3 (64 kDa) and distinct from mouse ZP3 (83 kDa) (Fig. 3). Since human spermatozoa do not normally bind to mouse zonae, it was reasoned that, if human ZP3 was incorporated into a chimaeric mouse–human zona pellucida, then human spermatozoa would bind to the ‘humanized’ zona if ZP3 were solely responsible for sperm binding.

The transgenic lines expressing human ZP3 were bred into the *Zp3*-null line to eliminate endogenous mouse ZP3 that might compromise the interpretation of results. The expression of huZP3 protein in mouse oocytes was able to rescue the mouse ZP3-null phenotype both structurally (Fig. 2b) and functionally. Despite modifications after translation that are



**Fig. 4.** Assay of mouse and human sperm binding to eggs from normal and huZP3 rescue mice. Ovulated eggs from normal (a, b) and huZP3 rescue mice (c,d) were incubated with motile mouse (a,c) or human (b,d) spermatozoa and washed to remove non-adherent spermatozoa. Despite the presence of human ZP3, human spermatozoa did not bind to the huZP3 rescue eggs (d), and, despite the absence of mouse ZP3 (c), mouse spermatozoa did bind to the huZP3 rescue eggs. The insets are control two-cell mouse embryos from each incubation.

distinctly different from those of mouse ZP3, human ZP3 is incorporated into an extracellular matrix and effectively restores the integrity of the zona pellucida. Surprisingly, the re-creation of a zona matrix composed of moZP1, moZP2 and huZP3 also restored fertility in the huZP3 rescue mice. Furthermore, the presence of the human protein in the ‘humanized’ zonae was insufficient to support human sperm binding (Fig. 4) (Rankin *et al.*, 1998).

There are several possible explanations for these unanticipated results. First, modification after translation of human ZP3 expressed in mouse oocytes may convert the human protein to the functional equivalent of mouse ZP3. Although human ZP3 is modified in mice such that its molecular mass is indistinguishable from native human ZP3, these analyses do not preclude subtle modifications that may be critical for sperm–zona binding. Second, it is possible that human ZP3 by itself cannot support human sperm binding and that another zona protein or a combination of the zona proteins is required to recreate the three-dimensional structure recognized by human spermatozoa. Finally, a minor, as yet unidentified, component of the zona pellucida may be required for the specificity of sperm binding. Mouse lines in which each endogenous mouse protein is replaced by its human homologue are being established to investigate these possibilities and examine the effect

of single, as well as multiple, human zona proteins on fertility and sperm binding to 'humanized' zonae pellucidae.

### Conclusions

Despite 20 years of investigations in a variety of species, the molecular basis of sperm binding to the zona pellucida remains an enigma. It is clear that the zona matrix uses structural motifs that have existed for millions of years. Pre-eminent among these motifs is the zona domain present in all egg envelope proteins investigated to date, independent of the species or the site of zona protein synthesis. Outside this domain, the egg envelope proteins are more different, indicating that they provide functional domains unique for each species, while the zona domain serves as the core structural motif. Although a role for carbohydrates in mediating sperm binding to the zona pellucida have been widely embraced, genetic studies looking at specific candidates have not been confirmatory in mice.

While much emphasis has been placed on the participation of individual zona proteins in mediating sperm binding, less attention has been devoted to the supramolecular structure of the zona matrix. There are striking differences in sperm head morphology among vertebrates that may dictate corresponding variations in the zona matrices to accommodate effective sperm binding to the zona pellucida. The extent of the glycosylation of zona proteins varies substantially among vertebrates, even though the polypeptide backbones of individual classes of protein are well conserved. These differences in carbohydrate may affect the hydration of the zona mesh and, in conjunction with differences in protein structure, allow for substantial variation in the quaternary structure of the zona pellucida matrix.

It may be possible to address the role of individual proteins and that of the supramolecular structure experimentally using genetic approaches to complement biochemical and biological analyses *in vitro*. It seems particularly exigent to establish mouse lines in which genetic manipulations of the zona pellucida alter the specificity of sperm binding. If the supramolecular structure is of particular importance, then the establishment of mouse lines in which the endogenous mouse proteins are replaced with two and, eventually, all three human proteins, may result in human sperm binding to the 'humanized' zona pellucida. If specific protein or carbohydrate domains are responsible for sperm binding, it should be possible to establish mouse lines in which mutated mouse proteins preclude mouse sperm binding and are infertile *in vivo*. It remains surprising that the molecular basis of sperm-egg interactions, an essential first step in development, continues to mystify those who investigate the biology of fertilization.

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