

# Fertility and Taxon-Specific Sperm Binding Persist after Replacement of Mouse Sperm Receptors with Human Homologs

Tracy L. Rankin,<sup>1,5</sup> Jenell S. Coleman,<sup>1,3</sup>  
Olga Epifano,<sup>1</sup> Tanya Hoodbhoy,<sup>1</sup> Scott G. Turner,<sup>1</sup>  
Philip E. Castle,<sup>1,6</sup> Eric Lee,<sup>2</sup>  
Robert Gore-Langton,<sup>4</sup> and Jurrien Dean<sup>1,\*</sup>

<sup>1</sup>Laboratory of Cellular and Developmental Biology  
NIDDK

<sup>2</sup>Laboratory of Mammalian Genes and  
Development  
NICHD

National Institutes of Health

<sup>3</sup>Howard Hughes Medical Institute

<sup>4</sup>Department of Reproductive Medicine  
Suburban Hospital  
Bethesda, Maryland 20892

## Summary

The zona pellucida surrounding ovulated mouse eggs contains three glycoproteins, two of which (ZP2 and ZP3) are reported sperm receptors. After fertilization, the zona pellucida is modified *ad minimus* by cleavage of ZP2, and sperm no longer bind. *Crosstaxa* sperm binding is limited among mammals, and human sperm do not bind to mouse eggs. Using transgenesis to replace mouse ZP2 and/or ZP3 with human homologs, mouse lines with human-mouse chimeric zonae pellucidae have been established. Unexpectedly, mouse, but not human, sperm bind to huZP2 and huZP2/huZP3 rescue eggs, eggs fertilized *in vitro* with mouse sperm progress to two-cell embryos, and rescue mice are fertile. Also unanticipated, human ZP2 remains uncleaved after fertilization, and mouse sperm continue to bind early rescue embryos. These observations are consistent with a model in which the supramolecular structure of the zona pellucida necessary for sperm binding is modulated by the cleavage status of ZP2.

## Introduction

Mammalian fertilization occurs in the ampulla of the oviduct, where relatively few sperm (<100) encounter ovulated egg(s). Sperm, matured by passage through the female reproductive tract, bind to the extracellular zona pellucida surrounding the egg (Yanagimachi, 1994). Upon binding, the acrosome (a lysosomal-like structure on the anterior surface of the sperm head) vesiculates and releases lytic enzymes (Bedford, 1998). Sperm bind to the zona pellucida with some taxonomic specificity, and human sperm, which are particularly fastidious, will not bind to the zona matrix surrounding

mouse eggs (Bedford, 1977). After penetrating through the zona pellucida, a single sperm normally fuses with the egg plasma (vitelline) membrane to fertilize the egg. Shortly thereafter, cortical granules located in the egg's cortex fuse with the vitelline membrane and release their contents into the perivitelline space. One or more cortical granule components modify the zona pellucida, such that it becomes refractory to additional sperm binding and/or penetration (Wessel et al., 2001). While it is clear that the zona pellucida plays a vital role in fertilization and in the postfertilization block to polyspermy, our understanding of the roles of individual zona components in these carefully orchestrated events is less complete.

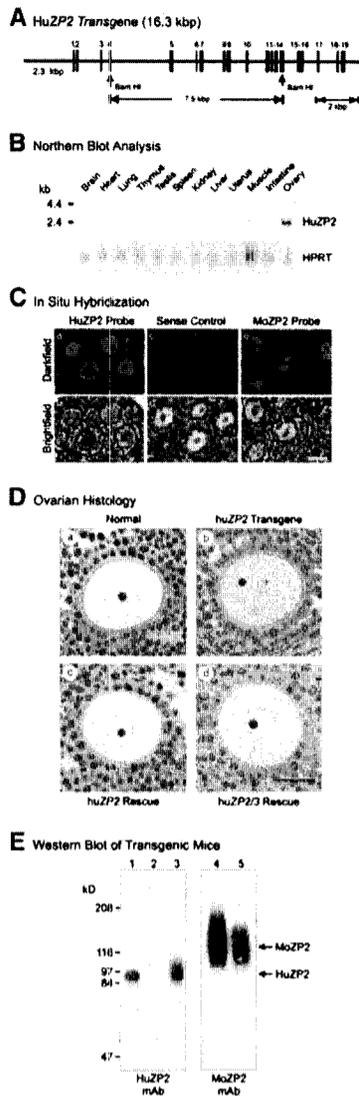
The mouse and human zonae pellucidae each contains three glycoproteins (ZP1 or ZPB, ZP2, and ZP3), which share common motifs (Rankin and Dean, 2000). Mouse ZP1 (623 amino acids) and human ZPB (540 amino acids) are the least conserved, sharing only 42% amino acid identity. This low degree of identity and reports in the rabbit and pig that the homologous protein plays a role in fertilization (Prasad et al., 1996; Yurewicz et al., 1998) raised the possibility that ZP1/ZPB is important for sperm binding. However, mice in which the single copy *Zp1* gene has been disrupted by targeted mutagenesis remain fertile, and mouse sperm bind a structurally defective zona matrix composed only of ZP2 and ZP3 (Rankin et al., 1999). ZP2 and ZP3, which are more conserved (61% and 67% amino acid identity, respectively) between mouse and human (Rankin and Dean, 2000), have been implicated as primary and secondary sperm receptors in mice (Wassarman, 1988).

Mice lacking ZP3 do not form a zona matrix and are infertile (Liu et al., 1996; Rankin et al., 1996). However, this phenotype can be rescued by the expression of human ZP3, which forms a zona pellucida matrix composed of mouse ZP1, mouse ZP2, and human ZP3. Surprisingly, human sperm do not bind to the chimeric human-mouse zona matrix, and, despite the absence of mouse ZP3, mouse sperm bind and fertilize the eggs (Rankin et al., 1998). More recently, we have established mouse lines lacking ZP2. Although females initially form a thin zona pellucida composed of ZP1 and ZP3, this fragile matrix is not sustained, and the resultant zona-free eggs are not fertilized *in vivo* (Rankin et al., 2001). We now report transgenic mice expressing human ZP2 and their use in establishing mouse lines with humanized zonae pellucidae, in which either human ZP2 by itself or together with human ZP3 replaces the endogenous mouse proteins and forms a zona matrix. Because human sperm will not bind to mouse eggs (Bedford, 1977), we reasoned that the replacement of an endogenous mouse protein with the corresponding human protein might change the specificity of sperm binding. Unexpectedly, we did not observe any alteration in the specificity of mouse and human sperm binding *in vitro*, and the mice with humanized zonae pellucidae are fertile *in vivo*.

\*Correspondence: jurrien@helix.nih.gov

<sup>5</sup>Present address: Reproductive Sciences Branch, Center for Population Research, NICHD, National Institutes of Health, Bethesda, Maryland 20892.

<sup>6</sup>Present address: Environmental Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892.



**Figure 1. Expression of Human ZP2 Transgene**

(A) A 16.3 kbp *Xho*I-*Not*I genomic fragment containing the entire *ZP2* genomic locus (1–19 exons) and 2.3 kbp of the 5' flanking region was injected into the pronuclei of one-cell zygotes to establish transgenic mouse lines expressing human ZP2. Transgenic mice were identified by digestion of genomic DNA with *Bam* HI (arrows) and Southern hybridization with <sup>32</sup>P-labeled human ZP2 cDNA. (B) Expression of human ZP2 was determined in transgenic line TgN(HuZP2)1NIH by Northern blot analysis. Total RNA (10 μg) from the indicated tissue was separated by 1% agarose/formaldehyde gel electrophoresis and transferred to a nylon membrane. HuZP2 transcripts were detected by autoradiography after hybridization of the membrane with <sup>32</sup>P-labeled human ZP2 cDNA. The blot was stripped and re-probed with <sup>32</sup>P-labeled human HPRT cDNA to assay the integrity of the loaded RNA. Molecular weight markers (kb) are indicated at left. (C) <sup>35</sup>S-labeled antisense RNA probes derived from human ZP2 (HuZP2 Probe [A and B]) and mouse ZP2 (MoZP2 Probe [E and F]) and sense RNA probes from human ZP2 (Sense Control [C and D]) cDNAs were hybridized to formaldehyde-fixed, paraffin-embedded ovarian sections from 3-week-old huZP2 transgenic mice. Sections were viewed either by darkfield (A, C, and E) or brightfield (B, D,

## Results

### Establishment of huZP2 and huZP2/huZP3 Rescue Mice

Three mouse lines expressing a human *ZP2* transgene (16.3 kbp) with the entire coding region and 2.3 kbp of 5' flanking sequences (Figure 1A) were established. Oocyte-specific expression was confirmed by Northern blot analysis (Figure 1B) and in situ hybridization of ovarian tissues (Figure 1C). The ovarian histology of transgenic mice expressing human ZP2 appeared normal (Figure 1Da and 1Db), and human ZP2 protein expressed in the transgenic mice had a molecular mass similar to native human ZP2 (90–110 kDa) and distinct from mouse ZP2 (120–140 kDa) (Figure 1E). These data suggest that the human ZP2 expressed in mouse eggs was posttranslationally modified as the native human ZP2, although these observations do not comment on the composition of the carbohydrate side chains and do not preclude minor glycosylation differences between native and transgenic human ZP2.

Mice lacking mouse ZP2 ovulate zona-free eggs and are infertile (Rankin et al., 2001). These *Zp2* null mice were crossed with the human ZP2 transgenic mice to establish mouse lines expressing mouse ZP1 (moZP1), human ZP2 (huZP2), and mouse ZP3 (moZP3), but not mouse ZP2 (moZP2). As observed with huZP3 rescue mice (Rankin et al., 1998), the replacement of moZP2 with huZP2 reconstituted a zona pellucida matrix (Figure 1Dc). In addition, by crossing the huZP2 rescue lines with the previously described huZP3 rescue line, we established mouse lines expressing human ZP2 and ZP3 in lieu of the two endogenous mouse proteins. Ovarian histology and the morphology of the zona pellucida matrix formed by moZP1, huZP2, and huZP3 appeared unremarkable (Figure 1Dd).

To confirm the composition of the zona pellucida from huZP2 and huZP2/huZP3 rescue mice, we stained ovulated eggs with monoclonal antibodies specific to the mouse and human zona proteins (Figure 2). As expected, eggs from normal female mice were surrounded by zonae pellucidae composed of moZP1, moZP2, and moZP3 (Figures 2A–C). The antibodies to human ZP2

and F). Both human and mouse ZP2 transcripts were detected in growing oocytes in huZP2 transgenic mice. Scale bar, 25 μm. (D) Plastic embedded ovarian sections (2 μm thick) containing growing follicles were stained with periodic-acid Schiff's reagent to highlight the zona pellucida surrounding oocytes with intact nuclei. The zona pellucida formed around growing oocytes from normal mice (A), transgenic mice expressing human ZP2 (B), huZP2 rescue mice (C), and huZP2/huZP3 rescue mice (D) were histologically similar. Thus, huZP2 and huZP3 can reconstitute a zona pellucida matrix in *Zp2* and *Zp3* null mice. Scale bar, 25 μm. (E) Immunoblot of human (4 eggs) or mouse (25 eggs) zonae pellucidae separated by SDS-PAGE (8% gel) and transferred to nitrocellulose membranes. Lane 1, nonviable human eggs; lanes 2 and 4, normal mouse eggs; lanes 3 and 5, transgenic mouse eggs. Lanes 1, 2, and 3 were probed with a monoclonal antibody (H2.8, 1:500) specific to human ZP2, and lanes 4 and 5 were probed with a monoclonal antibody specific to mouse ZP2 (IE-3, 1:1000). Immunoreactions for mouse and human ZP2 proteins were detected with horseradish peroxidase-conjugated secondary antibodies and chemiluminescence. Molecular weights (kDa) are indicated at the left.

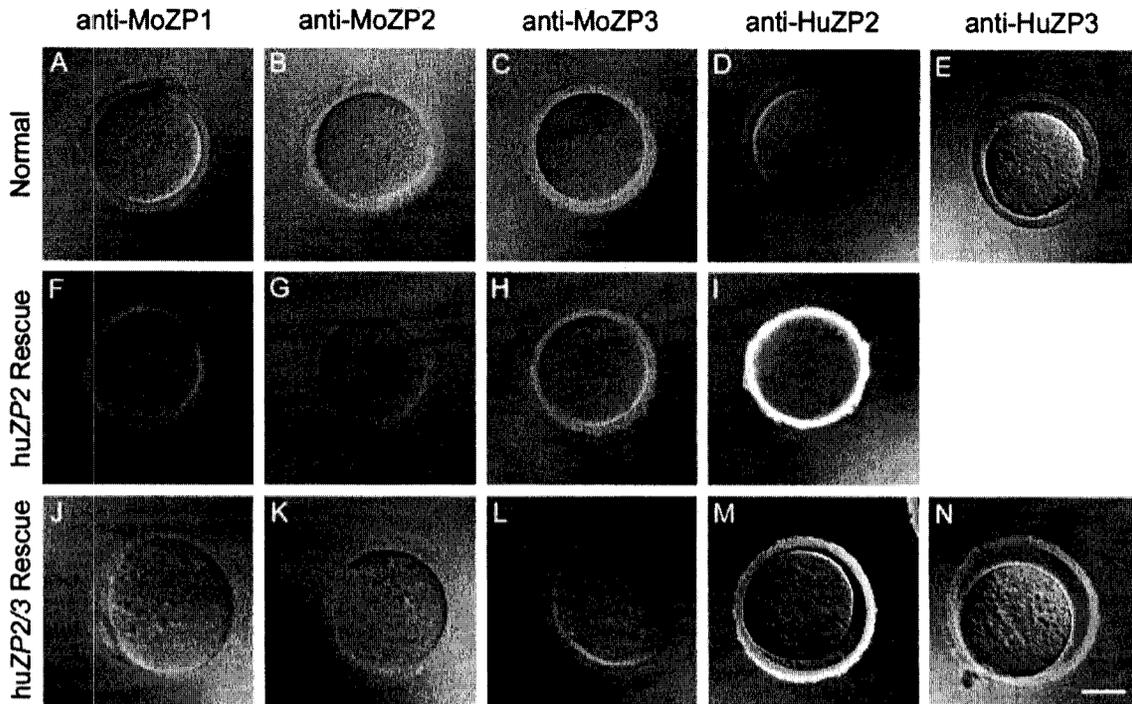


Figure 2. Protein Composition of Zonae Pellucidae from Normal, huZP2 Rescue, and huZP2/huZP3 Rescue Mice

Ovulated eggs from normal, huZP2 rescue, and huZP2/huZP3 rescue mice were stained with monoclonal antibodies specific to mouse ZP1 (A, F, and J), mouse ZP2 (B, G, and K), mouse ZP3 (C, H, and L), human ZP2 (D, I, and M), and human ZP3 (E and N). Zonae pellucidae surrounding ovulated eggs from normal mice contain mouse ZP1, ZP2, and ZP3, but not human ZP2 or ZP3. Zonae pellucidae from huZP2 rescue mice contain human ZP2, but not mouse ZP2. Zonae pellucidae from huZP2/huZP3 rescue mice contain both human ZP2 and ZP3, but neither mouse ZP2 nor ZP3. Scale bar, 25  $\mu$ m.

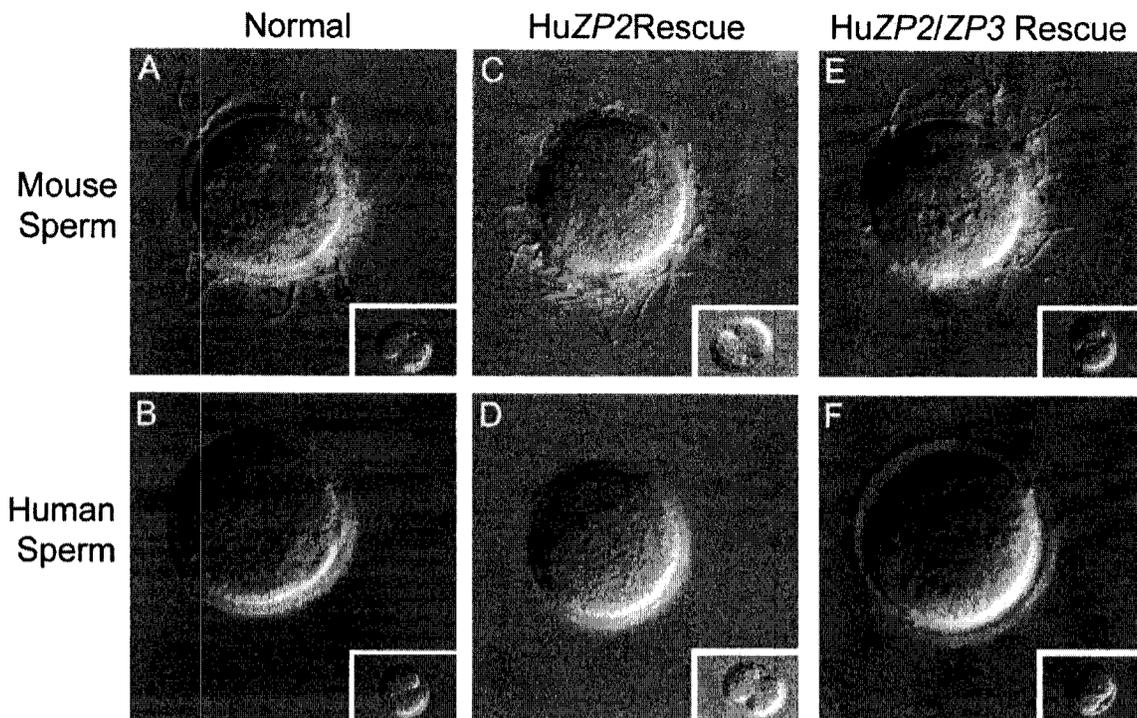
and ZP3 did not crossreact with the endogenous mouse proteins (Figures 2D and 2E). Zonae pellucidae from huZP2 rescue mice contained moZP1, huZP2, and moZP3, but not moZP2 (Figures 2F–2I), and those from the huZP2/huZP3 double rescue mice contained moZP1, huZP2, and huZP3, but neither moZP2 nor moZP3 (Figures 2K–2O). In each mouse line, both the human and mouse proteins were detected throughout the width of the humanized zona matrix, and no consistent abnormalities were observed in zonae pellucidae from the single and double rescue mice.

#### Fertility and Specificity of Sperm Binding in huZP2 and huZP2/huZP3 Rescue Mice

HuZP2 rescue and huZP2/huZP3 double rescue mice were freed from their cumulus masses and divided into two aliquots: one was assayed for mouse sperm binding, the other for human sperm binding. Capacitated mouse sperm bound avidly to normal eggs ( $25.1 \pm 1.8$  SEM/egg,  $n = 50$ ), whereas human sperm did not bind at all (Figures 3A and 3B). The ability of mouse sperm to bind to huZP2 rescue and huZP2/huZP3 double rescue eggs assured the functional integrity of their zonae pellucidae (Figures 3C and 3E). However, despite the presence of human ZP2 in the zona pellucida, human sperm did not bind to huZP2 rescue eggs (Figure 3D). Furthermore, even when both human ZP2 and ZP3 replaced the endogenous mouse proteins, human sperm still did not

bind to the humanized zona pellucida (Figure 3F). We further observed that, despite the absence of mouse ZP2 alone or both mouse ZP2 and ZP3, mouse sperm bound to huZP2 rescue ( $35.8 \pm 2.7$  SEM/egg,  $n = 32$ ) (Figure 3C) and huZP2/huZP3 double rescue eggs ( $69.9 \pm 5.0$  SEM/egg,  $n = 33$ ) (Figure 3E), even after washing with a pipette to remove all but 1–2 sperm from control two-cell mouse embryos (to which sperm will not bind physiologically). Thus, although the presence of human ZP2 (by itself or with human ZP3) in the transgenic zona pellucida was insufficient to support human sperm binding, the presence of the human zona proteins did not preclude *in vitro* mouse sperm binding.

To establish the physiologic relevance of these observations, we mated five huZP2 rescue, five huZP2/huZP3 double rescue, and five normal females with normal males. Although all rescue female mice were fertile, there was a decrease in the average litter size of huZP2 ( $5.7 \pm 0.7$  pups) and huZP2/huZP3 ( $4.2 \pm 0.6$  pups) rescue mice compared to normal ( $9.0 \pm 0.7$  pups) mice (Table 1). To determine whether this reflected diminished ovulatory capacity, females were primed with pregnant mare serum gonadotropin and stimulated to ovulate with human chorionic gonadotropin. HuZP2 and huZP2/huZP3 rescue females ovulated  $12.3 \pm 2.0$  and  $16.5 \pm 3.3$  eggs, respectively, compared to  $33.6 \pm 4.6$  eggs obtained from normal female mice (Table 1). These observations suggested abnormal developmental



**Figure 3. Ovulated Eggs from huZP2 and huZP2/huZP3 Rescue Mice Do Not Bind Human Sperm**

Ovulated eggs from normal (A and B), huZP2 rescue (C and D), and huZP2/huZP3 rescue (E and F) mice were incubated with  $5 \times 10^6$  motile, capacitated mouse (A, C, and E) or human (B, D, and F) sperm. After washing to remove nonadherent sperm, similar numbers of mouse sperm bound to normal ( $17.7 \pm 2.5$  SEM/egg), huZP2 rescue ( $15.7 \pm 5.2$  SEM/egg), and huZP2/huZP3 rescue ( $17.6 \pm 1.8$  SEM/egg) eggs, but human sperm did not bind to normal, huZP2 rescue, or huZP2/huZP3 rescue mice, even in the absence of washing. Insets, control two-cell mouse embryos from each incubation after washing.

competence of the germ cells in the rescue mice, as noted in *Zp2* and *Zp3* null mice (Rankin et al., 2001), which could account for decreased rates of *in vitro* fertilization (see below). Nevertheless, even though the fecundity was decreased in the rescue mice, it appeared that the replacement of endogenous mouse zona proteins with either human ZP2 by itself or both human ZP2 and ZP3 did not prevent fertility *in vivo*.

#### In Vitro Fertilization of huZP2 and huZP2/huZP3 Eggs

To further investigate the interaction between mouse sperm and humanized zonae pellucidae, we stimulated normal, huZP2 rescue, and huZP2/huZP3 double rescue females with gonadotrophins, and ovulated eggs in cumulus mass were isolated from their oviducts. Eight hours after insemination with  $>5 \times 10^6$  mouse sperm, zygotes derived from each genotype were scored for the presence of pronuclei. Twenty-four hours after insemination, the number of embryos that had progressed to the two-cell stage was determined (Figure 4). Fertilization and progression to the two-cell stage occurred in 82% of normal eggs, and the rate of polyspermy, as assayed by the presence of  $>2$  pronuclei and an inability to progress to the two-cell stage, was  $<3\%$  (383 eggs, four experiments). Even in the absence of washing, no

adherent mouse sperm were detected at the one- or two-cell stage of normal embryos at 8 and 24 hr after insemination, respectively (Figures 4A and 4B).

Although eggs obtained from huZP2 rescue and huZP2/huZP3 double rescue mice could be fertilized *in vitro*, the success rate was reduced. The number of pronuclei at the one-cell stage was difficult to ascertain because of persistent sperm binding (Figures 4C, 4D, 4G, and 4H), but, 24 hr after insemination, only 68% of huZP2 rescue eggs and 41% of the huZP2/huZP3 double rescue eggs had progressed to the two-cell stage (63 eggs, three experiments, and 123 eggs, two experiments, respectively). However, the most striking aspect of these experiments was continued mouse sperm binding to one- and two-cell embryos encased in zonae pellucidae containing human ZP2 in the presence of

**Table 1. Hormone-Induced Ovulation and Offspring**

	Eggs/Animal	Pups/Litter
Normal	$33.6 \pm 4.6$ (21) <sup>a</sup>	$9.0 \pm 0.7$ (16) <sup>b</sup>
Human ZP2 Rescue	$12.3 \pm 2.0$ (21)	$5.7 \pm 0.7$ (19)
Human ZP2/ZP3 Rescue	$16.5 \pm 3.3$ (15)	$4.2 \pm 0.6$ (22)

<sup>a</sup>Mean  $\pm$  SEM (number of animals) after stimulation with gonadotrophins.

<sup>b</sup>Mean  $\pm$  SEM (number of litters) after mating with normal males.

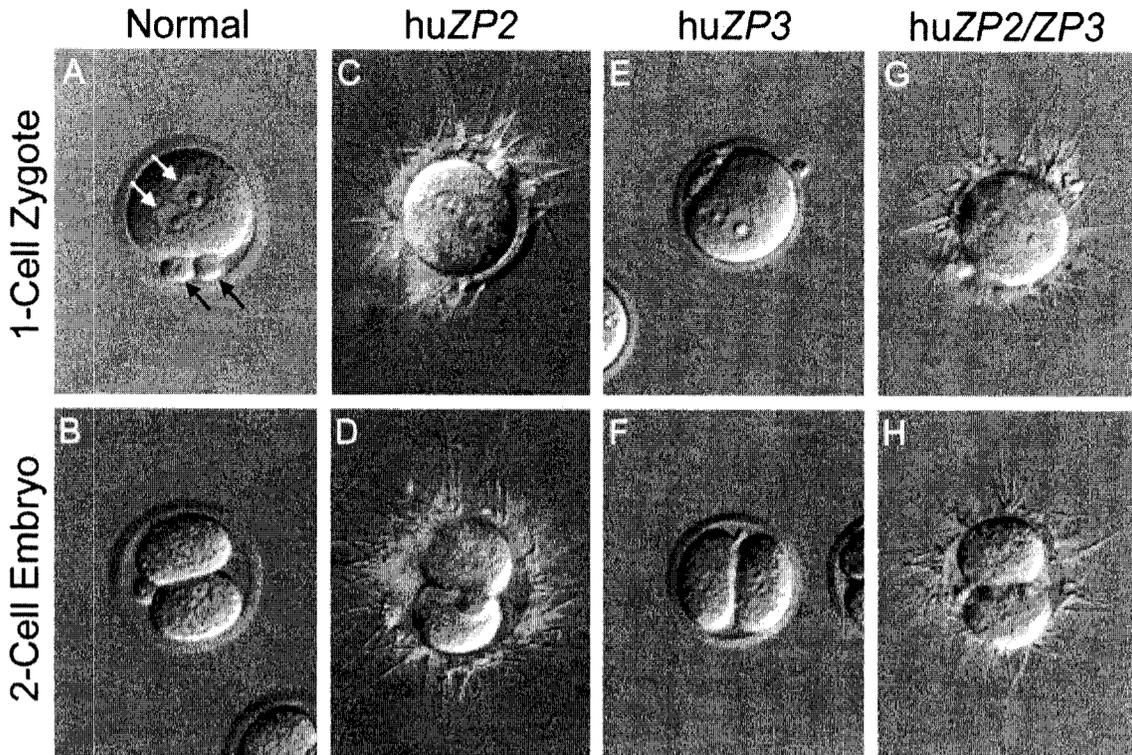


Figure 4. In Vitro Fertilization

Eggs in cumulus mass from normal (A and B), huZP2 rescue (C and D), huZP3 rescue (E and F), and huZP2/huZP3 double rescue (G and H) mice were examined morphologically 8 and 24 hr after insemination with capacitated, epididymal mouse sperm. Two pronuclei (white arrows) were detected in each of the four genotypes at 8 hr (A, C, E, and G), and cleavage to two cells occurred by 24 hr (B, D, F, and H). The persistence of sperm binding to the zona pellucida after in vitro fertilization was observed in huZP2 (C and D) and huZP2/huZP3 double rescue (G and H) mice, but not in normal (A and B) or huZP3 rescue (E and F) mice. Black arrows indicate polar bodies resulting from egg meiosis.

either human ZP3 (Figures 4G and 4H) or mouse ZP3 (Figures 4C and 4D). This binding persisted even after multiple washes with wide-bore pipettes. In vitro fertilization of huZP3 rescue eggs did not result in persistent sperm binding at either the one- or two-cell stage (Figures 4E and 4F) (Rankin et al., 1998). On occasion, supernumerary sperm (1–3) were observed in the perivitelline space between the inner aspect of the humanized zona pellucida and the plasma membrane of the eggs, as has been reported in normal eggs (Sato, 1979).

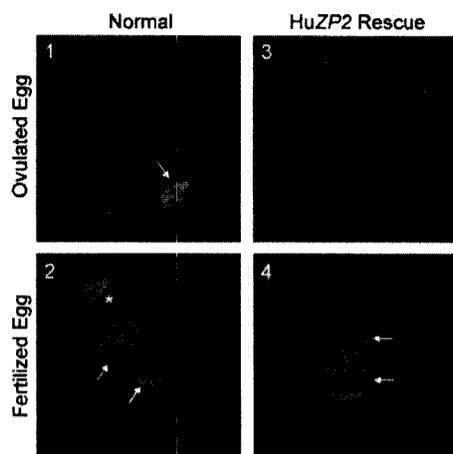
#### Cortical Granule Reaction and Postfertilization Modification of huZP2

After normal fertilization, peripherally located cortical granules exocytose their contents into the perivitelline space. These contents, although poorly characterized, are thought to modify the zona pellucida matrix to provide a postfertilization block to polyspermy, which prevents sperm binding and penetration of the zona pellucida. A possible explanation of the observed persistence of sperm binding to the humanized zona pellucida containing huZP2 would be the absence of a cortical granule reaction in the huZP2 rescue mice. In unfertilized normal

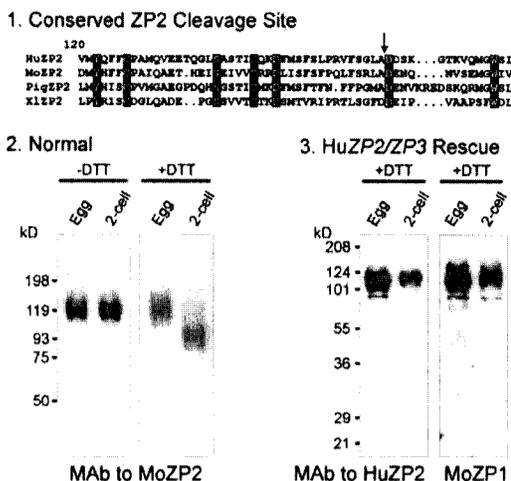
(Figure 5A1) and huZP2 rescue eggs (Figure 5A3), cortical granules were concentrated near the plasma membrane, with some staining scattered throughout the cytoplasm. The metaphase II chromosomes and spindle were visible (arrow), and the overlying cortex, encompassing almost 40% of the periphery, was devoid of cortical granules, as previously described (Ducibella et al., 1988; Deng et al., 2003). After mating with normal males, one-cell embryos derived from normal (Figure 5A) and huZP2 eggs (Figure 5A4) contained two pronuclei (arrows), but lacked cortical granules. The absence of cortical granules at the periphery was consistent with normal, postfertilization cortical granule exocytosis.

The exocytosis of cortical granules in normal mouse embryos correlates temporally with the postfertilization proteolytic cleavage of mouse ZP2 (120 kDa) into two fragments (90 kDa and 30 kDa), which is apparent on SDS-PAGE only under reducing conditions (Moller and Wassarman, 1989). The ZP2 cleavage site has been biochemically documented in pig, cow, and *Xenopus laevis* and is conserved in mouse and human (Figure 5B1). Consistent with cleavage between Ala<sub>167</sub> and Asp<sub>188</sub> of mouse ZP2, a monoclonal antibody specific to amino acids 114–129 binds to the smaller, N-terminal, cleaved

**A Cortical Granule Reaction**



**B Western Blot Analysis**



**Figure 5. Human ZP2 Remains Intact in Two-Cell Embryos**

(A) Cortical granule reaction occurs in huZP2 rescue mice. Unfertilized eggs or one-cell embryos were obtained after in vivo fertilization from normal (1 and 2) and huZP2 rescue (3 and 4) mice. After permeabilization, eggs and embryos were stained with rhodamine-conjugated LCA to image cortical granules at the equatorial plane, which were present in eggs (1 and 3), but not in one-cell embryos (2 and 4). Arrows indicate metaphase chromosomes or pronuclei stained with DAPI and present in eggs and embryos, respectively. Asterisks indicate polar bodies. The metaphase spindle in the normal egg was stained with a fluorescein-conjugated antibody specific to microtubules.

(B) Immunoblot analysis. (1) The N-terminal region of human ZP2 (amino acid residues 120–155) that contains the proteolytic cleavage of ZP2 (arrow) associated with the postfertilization block to polyspermy is conserved. The cleavage site has been determined in *Xenopus* (upstream of DE, residues 157–158) and pig (DE, 169–170), and the diacidic site is conserved in mouse (DE, 168–169) and human (DD, 173–174) ZP2. Identical amino acids in these four species are shaded. (2) Immunoblot analysis of 15 eggs and two-cell embryos from normal mice with mAb specific to the C terminus of mouse ZP2. Ovulated eggs (eggs) and two-cell embryos (2-cell) were incubated with (+DTT) or without (–DTT) dithiothreitol prior to SDS-PAGE. (3) Immunoblot analysis of eight eggs and two-cell embryos from huZP2/huZP3 double rescue mice after treatment with DTT with an mAb specific to human ZP2 (lanes 1 and 2) or after stripping (50°C, 30 min, 2% SDS, 0.1 M β-mercaptoethanol, and 62.5 mM Tris-HCl [pH 6.7]) to mouse ZP1 as a load control. The mobility of human ZP2 (Bauskin et al., 1999) and mouse ZP1 (Bleil and Wassarman, 1980b; Shimizu et al., 1983) run under reducing conditions correspond to 90–110 kDa and 120 kDa, respectively.

peptide fragment under reducing conditions (Greenhouse et al., 1999). A similar cleavage has been observed in human ZP2, which is present as a 90–110 kDa protein when isolated from eggs and as an ~65 kDa proteolytic fragment when isolated from embryos and analyzed under reducing conditions (Bauskin et al., 1999).

To examine the postfertilization processing of human ZP2, we harvested ovulated eggs and two-cell embryos from normal and huZP2/huZP3 mice. Each sample was divided; one-half was analyzed by SDS-PAGE without further treatment, and the other half was reduced with 5 mM dithiothreitol. Mouse ZP2 was detected as a 120 kDa band in control ovulated eggs and two-cell embryos under nonreducing conditions. Reduction of disulfide bonds did not affect the mobility of ZP2 in ovulated eggs (120 kDa) but changed the apparent mobility of ZP2 in two-cell embryos to 90 kDa, as previously reported (Figure 5B2). However, no change in the apparent molecular mass of human ZP2 (90–110 kDa) was observed in eggs and two-cell embryos derived from huZP2 rescue and huZP2/huZP3 double rescue mice before (data not shown) or after reduction with dithiothreitol (Figure 5B3, lanes 1 and 2). Comparable amounts of zonae pellucidae were present in each lane, as indicated by stripping (50°C, 30 min in 2% SDS, 0.1M β-mercaptoethanol, and 62.5 mM Tris-HCl [pH 6.7]) the membrane and reprobing with a monoclonal antibody specific to mouse ZP1 (Figure 5B3, lanes 3 and 4).

**Discussion**

Despite extensive investigation, there is no unifying model for the molecular basis of sperm binding to the zona pellucida that results in mammalian fertilization. Both mouse and human zonae pellucidae are composed of three major glycoproteins, ZP1/ZPB, ZP2, and ZP3. Mice lacking ZP1 have a structurally abnormal zona matrix, but mouse sperm bind and fertilize *Zp1* null eggs both in vitro and in vivo (Rankin et al., 1999). Thus, if mouse sperm binding is mediated by a single protein, it is unlikely to be ZP1. Both *Zp2* (Rankin et al., 2001) and *Zp3* (Liu et al., 1996; Rankin et al., 1996) null mice are sterile, but the absence of a zona pellucida matrix surrounding ovulated eggs from these mouse lines precludes analysis of the import of either protein in sperm binding. By introducing human ZP2 or human ZP3 into transgenic mice and crossing them into the corresponding null background, human ZP2 (this manuscript), human ZP3 (Rankin et al., 1998), and human ZP2/ZP3 (this manuscript) rescue mouse lines have been established. In each case, the presence of the human protein(s) reconstitutes the zona pellucida. However, despite the presence of human ZP2 and human ZP3 (individually or together), human sperm do not bind to the humanized zona matrix. Conversely, in the absence of mouse ZP2, mouse ZP3, or both, mouse sperm bind and the huZP2 rescue, huZP3 rescue, and huZP2/huZP3 double rescue

lines are fertile in vivo. These data suggest that sperm interactions with the zona pellucida matrix are independent of binding to individual, taxon-specific ZP2 or ZP3 proteins and are unanticipated, given the accepted roles of mouse ZP2 (Bleil et al., 1988) and ZP3 (Bleil and Wassarman, 1980a) as sperm receptors.

#### Mouse Sperm Binding

Previously, individual zona proteins have been isolated by SDS-PAGE and assayed for sperm receptor activity. ZP3 (but not ZP1 or ZP2) inhibits in vitro sperm binding to ovulated eggs in a dose-dependent manner (Bleil and Wassarman, 1980a). This inhibitory activity has been ascribed to O-linked carbohydrate side chains attached to serine residues at positions 332 and 334 of the ZP3 polypeptide chain (Florman and Wassarman, 1985; Chen et al., 1998). The further observation that ZP3 (or ZP3 glycopeptides) isolated from two-cell embryos does not inhibit sperm binding led to a model of sperm binding based on the oligosaccharide side chains of a single zona protein. To wit, mouse sperm bind to O-glycans attached to Ser332 and Ser334 of ZP3, and the postfertilization release of these carbohydrate moieties by cortical granule glycosidases results in the inability of sperm to bind to the zona pellucida surrounding two-cell embryos (Wassarman, 2002). However, microscale mass spectrometry of native mouse zonae pellucidae does not detect O-glycan occupancy of either Ser332 or Ser334 (Boja et al., 2003), and mutant ZP3 (Ser332 → Gly332; Ser334 → Ala334) transgenic mice lacking linkage sites for the implicated O-glycans remain fertile (Liu et al., 1995), although definitive assessment of reproductive fitness in the *Zp3* null background has not been reported. An alternative explanation of these data may lie in the reported ability of solubilized ZP3 to trigger the acrosome reaction and the observation that acrosome-reacted sperm do not bind to the zona pellucida (Saling et al., 1979). Although entertained, this latter hypothesis was considered less likely than the former (Bleil and Wassarman, 1980a, 1983) and would not explain the observations that ZP3 glycopeptides inhibit sperm binding (Florman and Wassarman, 1985) but do not induce the acrosome reaction (Florman et al., 1984; Leyton and Saling, 1989).

In light of these earlier reports, a possible explanation of mouse sperm binding to huZP2, huZP3, and huZP2/huZP3 rescue eggs could be that human proteins expressed in mouse oocytes are functionally converted to mouse specificity by posttranslational modifications. Even though the primary structures of human and mouse ZP2 and ZP3 proteins are conserved, posttranslational modifications (primarily glycosylation) result in significant size differences of the native proteins (Bleil and Wassarman, 1980b; Shabanowitz and O'Rand, 1988). While many posttranslational modifications are determined by primary protein structure and secondary folding, there could be differences in the glycosylation specificity in mouse and human oocytes. However, the molecular masses of human ZP2 and ZP3 expressed in mouse oocytes remain similar to native human proteins (90–110 kDa and 64 kDa, respectively) and distinct from mouse proteins (120 kDa and 83 kDa, respectively) (this manuscript; Rankin et al., 1998). These observations

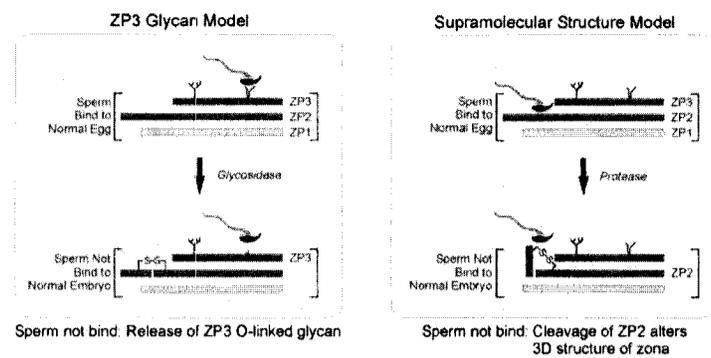
suggest that the primary structure of the human and mouse ZP2 and ZP3 proteins is the major determinant of glycosylation in mouse oocytes and that the human zona proteins are modified quite differently from the endogenous mouse proteins.

Nevertheless, there could be subtle posttranslational modifications that occur in mouse eggs that control the specificity of sperm binding. Two terminal O-linked oligosaccharides on ZP3 have been suggested to mediate mouse sperm binding. One is a terminal  $\alpha$ 1,3 galactose (Bleil and Wassarman, 1988) and the other is a terminal N-acetylglucosamine proposed to bind  $\beta$ 1,4 galactosyl transferase, a putative sperm receptor (Miller et al., 1992). A cortical granule N-acetylglucosaminidase has been implicated in the release of the latter (Miller et al., 1993), although there is no direct biochemical evidence of postfertilization modification of mouse ZP3. Furthermore, the continued fertility of mutant mice lacking either the enzyme that adds terminal  $\alpha$ 1,3 galactose (Thall et al., 1995; Liu et al., 1997) or  $\beta$ 1,4 galactosyl transferase (Lu and Shur, 1997; Asano et al., 1997) makes it unlikely that these are the sole determinants of mouse sperm binding.

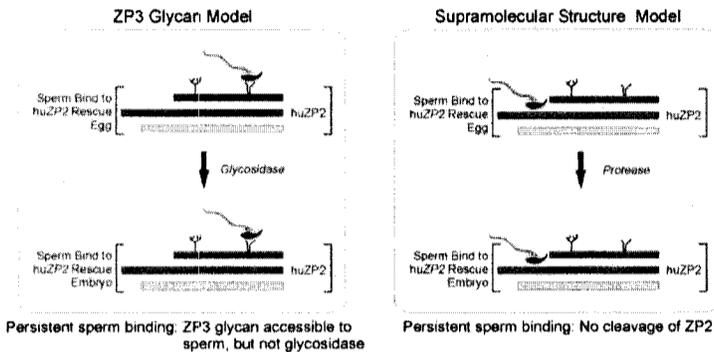
In this context, the continued binding of sperm to fertilized eggs from huZP2 and huZP2/huZP3 rescue mice is difficult to reconcile with posttranslational modifications of an individual zona protein serving as the primary site for sperm binding. If mouse sperm bind to the humanized zona pellucida because of mouse-specific glycosylation of an individual human zona protein, then the carbohydrate side chain should be released by the cortical granule reaction to account for the absence of postfertilization sperm binding. However, despite the occurrence of the cortical granule reaction in eggs with humanized zonae containing huZP2, mouse sperm binding persists after fertilization. Although the supramolecular structure of the humanized zona pellucida may differ from normal, it is difficult to envision a mouse-specified carbohydrate side chain that would be accessible for persistent, postfertilization sperm binding, yet unavailable for release by a mouse cortical granule glycosidase (Figure 6).

The genetic data from mutant mouse lines support an alternative explanation of the molecular basis of sperm binding to ovulated eggs (Figure 6). These observations are consistent with a model in which intact ZP2 regulates the three-dimensional structure of the zona matrix formed by the constitutive glycoproteins. Under this formulation, the supramolecular structure of the zona pellucida normally supports sperm binding to the zona pellucida surrounding ovulated eggs (the molecular bases of these interactions may involve protein, carbohydrate, or both). Following successful fertilization, cortical granules release (ad minimus) a protease that cleaves ZP2 within the zona matrix. This simple cleavage results in an alteration of the supramolecular structure of the zona pellucida and renders it unable to support sperm binding. Consistent with this model, isolated cortical granule exudates, which normally cause a dramatic decrease in sperm binding to ovulated eggs, do not do so in the presence of protease inhibitors (Barros and Yanagimachi, 1971; Gwatkin et al., 1973; Wolf and Hamada, 1977). Although not precluded in this hypothesis, loss of any constituent portions of the zona (carbohydrate

### A Normal Mice



### B HuZP2 Rescue Mice



side chains or protein) need not be postulated. This model accounts for the persistence of sperm binding to the humanized zona pellucida containing intact human ZP2 and predicts that rescue of *Zp2* null mice with mouse ZP2 mutated to preclude postfertilization cleavage would result in a similar phenotype.

#### Postfertilization Block to Sperm Penetration

After normal mouse fertilization, there are two potent blocks to polyspermy: the vitelline membrane of eggs become refractory to sperm fusion, and additional sperm do not bind or penetrate the zona pellucida (Yanagimachi, 1994; Wessel et al., 2001). The molecular basis of the former remains obscure (Jaffe et al., 1983; Horvath et al., 1993), but the latter is associated with the egg's cortical granule reaction (Barros and Yanagimachi, 1971). Whether the block requires enzymatic modification of the zona pellucida (Moller and Wassarman, 1989; Miller et al., 1993) or the imposition of a physical barrier (Dandekar and Talbot, 1992; Green, 1997) is unknown, and some mammals (e.g., rabbits) do not have an effective zona block to sperm penetration (Gould et al., 1971). The only documented modification of the zona pellucida that temporally correlates with the zona block in mice and humans is cleavage of ZP2, which results in an N-terminal peptide (~30 kDa) that remains covalently linked to the parental fragment via a disulfide bond(s) (Moller and Wassarman, 1989;

Figure 6. Models of Fertilization

(A) In the ZP3 glycan model, oligosaccharide side chains linked to ser332 and ser334 of ZP3 act as primary sperm receptors and their release by cortical granule glycosidase(s) following fertilization account for the inability of sperm to bind to fertilized eggs. In the supramolecular structure model, the zona glycoproteins form a three-dimensional matrix to which sperm can bind. After fertilization, a cortical granule protease(s) cleaves ZP2 and alters the supramolecular structure of the zona pellucida, rendering it unable to support sperm binding.

(B) The postfertilization persistence of sperm binding to humanized zonae pellucidae containing huZP2 is difficult to reconcile with the ZP3 glycan model. According to this model, O-linked oligosaccharides would remain accessible after fertilization for sperm binding and yet be inaccessible for cleavage by glycosidase(s) released by the cortical granule reaction. However, the persistence of sperm binding is consistent with the supramolecular structure model, in which intact huZP2 preserves prefertilization structures that support continued sperm binding, even after the cortical granule reaction. Why human ZP2 remains uncleaved by the mouse cortical granule protease remains to be determined.

Bauskin et al., 1999). However, the current results suggest that ZP2 cleavage is not required to block sperm penetration.

The presence of only two pronuclei in one-cell embryos, their progression to the two-cell stage in vitro, and the birth of live pups attest to an effective block to polyspermy in huZP2 and huZP2/huZP3 rescue embryos. Even though sperm continue to bind after fertilization, the absence of excessive sperm (Sato, 1979) in the perivitelline space suggests that part of this block is attributable to the zona pellucida. If correct, the presence of intact huZP2 after fertilization suggests that the zona block observed with the humanized zona pellucida may be independent of huZP2 cleavage. The ZP2 cleavage site, molecularly defined in pig and *Xenopus laevis* as an aspartate-glutamate site (Hasegawa et al., 1994; Tian et al., 1999), appears conserved in mouse and human ZP2, and it is therefore unclear why human ZP2 remains intact in the chimeric zona pellucida. Either the local sequence (with cleavage at a diaspertate site) of human ZP2 differs sufficiently from mouse ZP2 (with cleavage at an aspartate-glutamate site), rendering the responsible mouse protease nonfunctional, or the cleavage site is hidden by an altered supramolecular structure of the chimeric mouse-human zona pellucida matrix. Although fecundity is decreased, the presence of two-cell embryos and live pups from huZP2 and huZP2/huZP3 females implies that the combined integrity of the zona and plasma membrane blocks are sufficient to

prevent polyspermy, leading to polyploidy and embryonic lethality.

#### Human Sperm Binding

The absence of human sperm binding to the huZP2/huZP3 double rescue mice indicates that human ZP2 and ZP3 proteins are not sufficient to support human sperm binding and raises the possibility that an additional human zona protein(s) is required. As described, mouse ZP1 (623 amino acids) and human ZPB (540 amino acids) are the least conserved (53% similar and 42% identical amino acids) among the three zona proteins (Rankin and Dean, 2000). Although the continued fertility of *Zp1* null mice indicates that mouse ZP1 is not required for mouse sperm binding, the differences between mouse ZP1 and human ZPB may enforce structural constraints in the zona matrix that are critical for sperm recognition in a taxon-specific manner. Alternatively, sperm binding to humanized zonae pellucidae may require that all three zona proteins come from the same species. Each hypothesis can be addressed experimentally by expressing human ZPB in the mouse *Zp1* null background and then crossing it into the human ZP2/ZP3 rescue line to establish triple human ZPB/ZP2/ZP3 rescue mice.

There is also *in silico* evidence of a fourth human zona protein. An analysis of data compiled from the Human Genome Project identified a potential human *ZP1* gene that encodes a 638-amino acid protein similar in size and more homologous to mouse ZP1 (623 amino acids, 67% identity) than to human ZPB (540 amino acids, 42% identity) (Hughes and Barratt, 1999). Although the presence of transcripts in the human EST database suggests that the *ZP1* gene is transcribed (testes, brain, and kidney), neither oocyte nor ovarian expression has been reported. Because mouse ZP1 is not required for mouse sperm binding and human sperm do not bind to mouse eggs, it seems probable that, if the newly conceptualized human *ZP1* is involved in human sperm binding, it functions in conjunction with other zona proteins. If true, this would be consistent with earlier observations that human sperm (needing both ZP1 and ZPB proteins under this formulation) will not bind to mouse eggs, but mouse sperm (needing neither) bind to human eggs (Bedford, 1977).

#### Conclusion

Taken together, these genetic data indicate that no single mouse zona pellucida protein is obligatory for taxon-specific sperm binding (i.e., *moZp1* null, huZP2 rescue, and huZP3 rescue mice are fertile) and that two human proteins (ZP2 and ZP3) are not sufficient to support human sperm binding. The role of carbohydrate side chains as mediators of sperm binding to a single protein in the zona pellucida has been widely embraced. Intrinsic to this hypothesis is that the absence of postfertilization sperm binding results from cleavage of the receptor sugars by cortical granule glycosidases. However, this model does not anticipate the observed sperm attachment to chimeric zonae pellucidae (containing human ZP2) after successful fertilization and completion of the cortical granule reaction. In particular, it is difficult to

visualize a mouse-specified carbohydrate side chain attached to a single zona protein (mouse or human) that would be accessible and account for continued sperm binding, yet unavailable for release by a cortical granule glycosidase. An alternative hypothesis is that the preferential zona pellucida with intact ZP2 has a supramolecular structure that is permissive for sperm binding. The normal postfertilization cleavage of ZP2 would modify the three-dimensional structure, making it nonpermissive for sperm binding without an obligatory loss of constitutive zona components. If this hypothesis is correct, the intact human ZP2 observed in the humanized zona pellucida of the early embryo would maintain the preferential supramolecular structure and account for continued sperm binding. Whether the polypeptide backbones or the carbohydrate side chains have primacy in defining the supramolecular structures to which sperm bind remains to be determined.

#### Experimental Procedures

##### Construction of huZP2 Transgene and Generation of Transgenic Mice

A human placental genomic library in pWE 15 (Stratagene, La Jolla, CA) was successfully screened with <sup>32</sup>P-labeled human ZP2 cDNA (Liang and Dean, 1993) for clones containing intact human ZP2. A 16.3 kbp XhoI-NotI genomic fragment containing the entire locus (19 exons) and 2.3 kbp of the 5' flanking region was isolated by gel electrophoresis (0.7% agarose), purified (QIAEX II Gel Extraction Kit; QIAGEN), and dissolved (1 ng/ml) in injection buffer (10 mM Tris-HCl [pH 7.5] and 0.1 mM EDTA). DNA (5–10 pl) was injected into the male pronucleus of one-cell embryos FVB/N, which were subsequently transferred to the oviducts of foster mothers (NIH Swiss). Three transgenic lines were established, TgN(HuZP2)1–3NIH.

To detect transgenic animals, we isolated DNA from 1 cm of mouse tail at P21 and resuspended it in 150 μm buffer (Rankin et al., 1996). An aliquot (30 μm) was digested with BamHI, separated by gel electrophoresis (0.8% agarose), and transferred onto a nylon membrane (Schleicher & Schuell). The membrane was hybridized with a 1.4 kb BamHI cDNA fragment (encoding exons 9–14) labeled with [<sup>32</sup>P]dCTP (Ready-To-Go DNA; Pharmacia BioTech, Piscataway, NJ) (Rankin et al., 1996). Hybridization signals were detected by autoradiography. HuZP2 transgenic mice were bred with *Zp2* null (Rankin et al., 2001) and human *ZP3* rescue (Rankin et al., 1998) mice to establish huZP2 and huZP2/huZP3 rescue lines. Ovaries were isolated from 6- to 8-week-old normal, huZP2 transgenic, huZP2 rescue, and huZP2/huZP3 rescue females, fixed in 3% glutaraldehyde, embedded in methacrylate, and stained with periodic acid Schiff's reagent and hematoxylin (Rankin et al., 1999).

##### Expression of the huZP2 Human Transgene

For northern blot analysis, total RNA (10 μg) isolated from 4- to 6-week-old mouse tissues with RNazolB (Tel-Test) was separated by 1% agarose/formaldehyde gel electrophoresis and transferred to a nylon membrane (Schleicher & Schuell). The membranes were sequentially hybridized with human ZP2 cDNA (1–2266 bp) and HPRT cDNA (672–1276 bp) labeled with <sup>32</sup>P-dCTP (Rankin et al., 1996). Hybridization signals were detected by autoradiography. Ovaries from 12-day-old females were isolated and prepared for *in situ* hybridization (Rankin et al., 1998). Full-length human ZP2 cDNA and mouse ZP2 cDNA were linearized and used as a template for *in vitro* transcription of <sup>35</sup>S-UTP-labeled sense and antisense probes with T<sub>3</sub> and T<sub>7</sub> RNA polymerase (MAXIscript; Ambion). After 4 or 10 days of exposure, slides were developed with Dektol developer (diluted 1:1 with water) and Kodak fixer and counterstained with hematoxylin.

##### Immunoblotting Analysis

Monoclonal antibodies specific to human ZP2 (H2.8; mouse IgG<sub>1</sub>) and mouse ZP2 (M2c.2; rat IgG) were produced with hybridoma

techniques (Hildreth et al., 1989) with bacterially expressed, Ni<sup>2+</sup>-agarose purified, 6-histidine-tagged recombinant human ZP2 (amino acids 39–642) and SDS-PAGE-purified mouse zona pellucida proteins (Rankin et al., 1998), respectively, as immunogens.

Mouse eggs from 4-week-old normal and transgenic mice were isolated by superovulation with gonadotropins (Rankin et al., 1998), and nonviable human eggs were obtained under a protocol approved by the NIDDK and Suburban Hospital Institutional Review Boards. Eggs and two-cell embryos from normal, huZP2 rescue, and huZP2/huZP3 rescue mice were isolated to detect cleavage of ZP2 (Greenhouse et al., 1999). All cells were isolated in M2 media (Specialty Media) augmented with a protease inhibitor cocktail (Complete Mini; Roche) per instructions from the manufacturer and then quickly washed in Tris-buffered saline (100 mM Tris-HCl [pH 7.5] and 150 mM NaCl). Eggs/embryos were dissolved in sample buffer, separated either by 8% or 4%–20% gradient polyacrylamide SDS-PAGE, and transferred onto a nitrocellulose membrane for immunoblot analysis (Burnette, 1981). Blots were incubated with monoclonal antibodies specific to mouse ZP1, mouse ZP2 (East and Dean, 1984), or human ZP2 (1:500–1:1000, 1 hr, RT). Individual zona proteins were detected by biotin-conjugated goat anti-rat (for antibodies to mouse ZP1 and ZP2) or anti-mouse (for antibodies to human ZP2) IgG (1:1000; Jackson ImmunoResearch), after an enhancing step with Elite ABC Kit (Vectastain) and a chemiluminescence detection system (Amersham). Blots used more than once were stripped (50°C, 30 min in 2% SDS, 0.1M  $\beta$ -mercaptoethanol, and 62.5 mM Tris-HCl [pH 6.7]) before incubation with successive primary antibody.

#### Confocal Microscopy

Normal, huZP2 rescue, and huZP2/huZP3 rescue mice were stimulated with gonadotropins, and eggs or one-cell embryos were recovered 14–16 hr after administration of hCG from unmated and mated females, respectively (Rankin et al., 2001). To define the composition of the zona pellucida, ovulated eggs were fixed, stained with monoclonal antibodies to mouse ZP1, mouse ZP2, mouse ZP3, human ZP2, and human ZP3, and imaged by confocal microscopy (Rankin et al., 2001). To assess cortical granule status, embryos were fixed (2% paraformaldehyde in PBS, 1 hr, RT), washed in blocking solution (0.3% BSA, 100 mM glycine, 5  $\times$  5 min), and permeabilized (0.1% Triton X-100 in blocking solution). Samples were labeled with Rhodamine-LCA (20  $\mu$ g/ml in blocking solution, 45 min, RT) (Vector Laboratories) and washed in blocking solution (5  $\times$  5 min, RT). These samples were then incubated with an FITC-conjugated monoclonal antibody to  $\alpha$ -tubulin (15  $\mu$ g/ml in blocking solution, 1 hr, RT) (Sigma), washed again in blocking solution (8  $\times$  5 min), and mounted in Vectashield containing DAPI (Vector Laboratories). Control samples were not permeabilized but were otherwise treated the same.

Images were acquired with a Zeiss LSM 510 confocal microscope through a 63X Zeiss C-Apochromat water immersion objective (N.A. 1.2). Labeled antibodies specific to individual zona proteins were detected as follows: AMCA was excited with a 364 nm UV laser line, and emissions were detected through a 385 nm long-pass filter; TRITC was excited with a 543 HeNe laser, and emissions were detected through a 560 nm long-pass filter; and FITC was excited with the 488 nm line from an argon laser, and emissions were imaged through a 505 nm long-pass filter. Cortical granules labeled with Rhodamine-conjugated LCA were excited with a 543 HeNe laser, and emissions were detected through a 560 nm long-pass filter; DAPI-labeled DNA was excited with a 364 nm UV laser line, and emissions were detected through a 385–470 nm band-pass filter. Nomarski interference contrast images were obtained with a single-channel transmission detector.

#### Sperm Binding and In Vitro Fertilization

Ovulated eggs from normal, huZP2 rescue, and huZP2/huZP3 rescue female mice and two-cell embryos from normal females were isolated for mouse or human sperm binding assays (Rankin et al., 1998). Human sperm were obtained under an Institutional Review Board-approved protocol, in which suitable specimens were identified by successful human in vitro fertilization.

Normal, huZP2 rescue, and huZP2/huZP3 rescue eggs were inseminated with capacitated mouse spermatozoa in vitro (Rankin et al., 1999). All experiments were conducted in compliance with the guidelines of the Animal Care and Use Committee of the National Institutes of Health under a Division of Intramural Research, NIDDK-approved animal study protocol.

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