Human ZP3 restores fertility in Zp3 null mice without affecting order-specific sperm binding

Tracy L. Rankin1,*, Zhi-Bin Tong1,2, Philip E. Castle1, Eric Lee3, Robert Gore-Langton4, Lawrence M. Nelson2 and Jurrien Dean1

1Laboratory of Cellular and Developmental Biology, NIDDK, 2Developmental Endocrinology Branch, 3Laboratory of Mammalian Genes and Development, NICHD, National Institutes of Health and 4Department of Reproductive Medicine, Suburban Hospital, Bethesda, MD 20892, USA
*Author for correspondence (e-mail: tr44x@nih.gov)

SUMMARY

The mammalian zona pellucida surrounding ovulated eggs mediates sperm binding at fertilization, provides a postfertilization block to polyspermy, and facilitates passage of pre-implantation embryos down the oviduct. Although the three zona proteins (ZP1, ZP2, ZP3) are well conserved, mammalian fertilization is relatively specific and human sperm do not bind to the mouse zona pellucida. There are considerable in vitro data that ZP3 acts as a primary sperm adhesion molecule in mice and, by analogy, a similar role has been postulated for human ZP3. Genetically altered mice lacking ZP3 (Zp3<sup>3tm/3tm</sup>) do not form a zona pellucida and are infertile. To rescue this phenotype, transgenic mice expressing human ZP3 (67% identical to mouse ZP3) were produced and bred with Zp3<sup>3tm/3tm</sup> null mice. The resultant human ZP3 rescue females had chimeric zonae pellucidae composed of mouse ZP1, mouse ZP2 and human ZP3. Human ZP3 expressed in mouse oocytes had an apparent mass (64 kDa) indistinguishable from native human ZP3 and, by analogy, a similar role has been postulated for human ZP3. Genetically altered mice lacking ZP3 (Zp3<sup>3tm/3tm</sup>) do not form a zona pellucida and are infertile. To rescue this phenotype, transgenic mice expressing human ZP3 (67% identical to mouse ZP3) were produced and bred with Zp3<sup>3tm/3tm</sup> null mice. The resultant human ZP3 rescue females had

Key words: Order-specific sperm binding, Zona pellucida, Human fertilization, Mouse fertility, Sperm-egg interaction, Transgene, Mouse

INTRODUCTION

The members of a species consist of a discrete reproductive community that is variably maintained by pre-mating (habitat, mate discrimination, physiognomy), mating (fertilization) or postmating (hybrid lethality, sterility) determinants (O’Rand, 1988). Most commonly, species arise following physical separation and genetic evolution (allopatry) that results in reproductive isolation even when the two species are reunited (Coyne, 1992). Some species, however, arise from sympatric speciation in which distinct reproductive communities are formed in the absence of physical barriers (Schliewen et al., 1994; Shoemaker and Ross, 1996). Although the macromolecules that mediate sperm-egg interactions can serve as a cross-breeding block between species (Metz and Palumbi, 1996), this seems unlikely to be of critical importance for mammals which fertilize internally. Rather, the value of highly specific sperm-egg interaction to reproductive efficiency may lie in limiting egg access to a single sperm of the correct species and therefore, minimizing the risk of polyspermy and its lethal consequences. While the identification of mammalian zona binding proteins on sperm remains controversial (Snell and White, 1996), the zona pellucida surrounding eutherian eggs has a well-documented role in mediating specific sperm-egg interactions (e.g., Roldan and Yanagimachi, 1989). The mammalian zona pellucida is an extracellular matrix that surrounds growing oocytes, ovulated eggs and pre-implantation embryos. Although critical for normal folliculogenesis and for passage of the embryo down the oviduct, it has been most intensely investigated for its role in mediating fertility. In mammals, fertilization takes place in the ampulla of the oviduct where individual gametes fuse to form a one-cell zygote. Although tens of millions of sperm are deposited in the lower female genital tract, few progress as far as the oviduct. For successful fertilization, a motile sperm must penetrate three investments of the ovulated egg: the cumulus oophorus composed of cumulus cells interspersed in a matrix of hyaluronic acid; the extracellular zona pellucida surrounding the egg; and the egg plasma membrane, with which the sperm head fuses. The acrosome, a lysosomal-like structure at the anterior head of the sperm, must be intact for penetration through the cumulus oophorus and initial binding to the zona pellucida. The binding of sperm to the zona then causes the outer acrosomal and plasma membranes to fuse, creating fenestrations and releasing lytic enzymes that may modify the sperm surface, the zona pellucida, or both. Subsequently, the sperm penetrates the
zona pellucida, leaving a narrow oblique track, and enters the perivitelline space to fuse with the egg plasma membrane. Following fertilization, the zona pellucida is biochemically modified to prevent additional sperm from binding to, or penetrating, the zona matrix and therefore, provides a potent block to polyspermy (for review see, Yanagimachi, 1994).

In mice and humans, the zona pellucida is composed of three glycoproteins (ZP1, ZP2 and ZP3), the primary amino acid sequences of which were initially deduced from cDNA (Ringue et al., 1988; Chamberlin and Dean, 1990; Liang et al., 1990; Liang and Dean, 1993; Harris et al., 1994; Epifano et al., 1995b). Of the three zona proteins, mouse and human ZP3 are the most conserved, each having polypeptide chains of 424 amino acids which are 67% identical (77% similar). Mouse and human ZP2 contain 713 and 745 amino acids, respectively, which are 61% identical; whereas mouse ZP1 (623 amino acid) contain 83 additional amino acids compared to the human homologue (540 amino acids) and when aligned, only 43% of the amino acids are identical. Each zona protein is posttranslationally modified, and the apparent masses of the glycoproteins that participate in the insoluble zona matrix are quite different between the two species. The three mouse zona proteins are 180-200 kDa, 120-140 kDa, and 83 kDa for ZP1, ZP2 and ZP3, respectively (Bleil and Wassarman, 1980a; Shimizu et al., 1983); the three proteins in the human zona are 90-110 kDa, 64-78 kDa and 57-73 kDa, respectively (Sacco et al., 1981; Shabanowitz and O’Rand, 1988).

The specificity of sperm binding to heterologous zonae pellucidae differs among phylogenetic orders and the role of individual zona proteins in mediating sperm binding has been variously reported in mammals. Purified mouse ZP3 inhibits sperm binding to the zona pellucida and induces the sperm acrosome reaction, a pre-requisite to zona penetration. Mouse ZP2 is proposed to be a secondary sperm receptor, binding to acrosome-reacted sperm, and a current model suggests that ZP1 plays a structural role by cross-linking filaments of acrosome-reacted sperm, and a current model suggests that ZP3 functions as a zona transducer. A 6 kb fragment of mouse ZP3 extending from an EcoRI site in the 5' flanking region to an artificial KpnI site 9 bp from the transcription initiation site was subcloned into pBluescript KSII in which the A/jIIH-EcoRI region had been replaced by an oligonucleotide (5'-CATGTAAGATAACAGGTTAT-3') containing the restriction site for meganuclease I-SceI. An approx. 9 kb KpnI-Sall genomic fragment extending from -4 bp of the human ZP3 initiator ATG to exon 5 was ligated to human ZP3 CDNA extending from the same SalI site in exon 5 to the end of the cDNA and followed by 310 bp of DNA encoding the bovine growth hormone (BGH) polyadenylation and termination signals (pRc/CMV, Invitrogen). The resultant human ZP3 minigene was cloned into the KpmII-NorI sites of the plasmid containing the mouse ZP3 promoter and the final DNA construct was digested with I-SceI meganuclease and NotI restriction enzymes to release the approx. 16 kb human ZP3 transgene. The human ZP3 transgene was purified by agarose gel electrophoresis, isolated (GeneClean II, Bio 101) and dissolved in injection buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA) at 1 ng/ml. After injection of 5-10 pl into the male pronucleus of one-cell zygotes (FVB/N) (Hogan et al., 1989), the embryos were transferred to oviducts of foster mothers (NII Swiss).

Genotypes of resultant mice were determined by PCR of 5 μl of tail DNA (Rankin et al., 1996) using 3 oligonucleotides: 5'-CAGAT-GAGGTGTTTGAAGCCACAG-3' corresponding to the mouse Zp3 promoter (~113 to ~134 bp); 5'-CTGTGCTCTCATGGACAAGAAG-3' corresponding to exon 1 of human ZP3 (241 to 262 bp); 5'-CAGAGTCTGGAATTGACAGC-3' corresponding to exon 1 of mouse Zp3 (83-104 bp). The PCR reaction mixture was assembled in 25 μl with 0.5 mM of each primer according to the manufacturer's instruction (Life Technologies) and carried out in a Perkin Elmer/Cetus Thermal Cycler (Model 9600) (95°C for 5 minutes; 35 cycles of 95°C for 1 minutes, 60°C for 1 minutes, 72°C for 2 minutes; 72°C for 10 minutes). Plasmids containing mouse Zp3 genomic DNA (~8 to +3 kb) or the human ZP3 transgene served as templates for positive controls. PCR products diagnostic of the human ZP3 transgene (409 bp) and the endogenous mouse Zp3 (238 bp) were visualized by agarose gel electrophoresis (1.8%) containing ethidium bromide (0.1 μg/μl).

To produce human ZP3 rescue mice [Zp3tm/m, TgN(HuZP3)], male Zp3tm1Nih/m1Nih mice (Rankin et al., 1996) were crossed with female mice that carry and express the human ZP3 transgene, TgN(HuZP3). Those mice in the F1 generation that were Zp3tm1Nih/m1Nih were crossed with female mice that express the human ZP3 transgene, TgN(HuZP3). The mice in the F1 generation that were Zp3tm1Nih/m1Nih were crossed to produce the Zp3tm3Nih, TgN(HuZP3) F2 mice. The latter mice also expressed ZP3 (238 bp) as diagnostic of the human ZP3 transgene (409 bp) and the endogenous mouse Zp3 and ZP2 (180-200 bp) were expressed as described previously (Tong et al., 1997). The Southern blot hybridizations were performed using a RPA II kit (Ambion) as described previously (Tong et al., 1997). Human ZP3 cDNA (319-731 nt) was used as a template for preparation of an antisense RNA probe which was synthesized in vitro using T7 RNA polymerase and [α-32P]UTP (3000 Ci/mmol, ICN) according to the MAXIscript kit protocol. The probe and protected RNA fragment

**MATERIALS AND METHODS**

**Transgenic mouse lines**

A 6 kb fragment of mouse Zp3 extending from an EcoRI site in the

---

**T. L. Rankin and others**

---
sections were then incubated overnight at 4°C with monoclonal antibody specific to mouse ZP1 and human ZP3, respectively, were produced with hybridoma techniques (Hildreth et al., 1989) using gel-purified mouse ZP1 protein (Epifano et al., 1995b) and a human ZP3 peptide<sup>335-350</sup> with a C-terminal cysteine (RQPHVMSQWSRS-<sup>342</sup>) coupled to keyhole limpet haemocyanin (Pierce) as immunogens. The specificity of the two antibodies was confirmed by their ability to react only with the target zona protein on western blots. Monoclonal antibodies IE-3 (rat IgG) and IE-10 (rat IgG) are specific to mouse ZP2 (East and Dean, 1984) and ZP3 (East et al., 1985), respectively. For immunohistochemistry, ovaries from 3-week-old females were fixed overnight in Bouin's fixative, dehydrated through graded alcohol solutions and embedded in paraffin (American Histolabs).

Mouse zonae pellucidae were isolated from ovaries from 9 transgenic or normal, litter-mates (3- to 6-week-old) by homogenization and centrifugation through a Percoll gradient (Bleil et al., 1988). After heat solubilization (60°C, 15 minutes), half of the sample was analyzed by SDS-PAGE using a 9% acrylamide gel and visualized by silver staining (Shimizu et al., 1982).

Ovaries were isolated from 3-week-old females, fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 for 3-16 hours, rinsed in the same and transferred to 70% ethanol. Tissues were dehydrated, embedded in methacrylate and 2 μm sections were mounted for staining with periodic-acid Schiff's reagent (PAS) and hematoxylin (American Histolabs).

Minimal 6 kb of the mouse Zp3 gene were detected (Fig. 1B). Only the endogenous mouse Zp3 gene showed good to excellent progression. Human sperm were incubated at 37°C for up to 2 hours in Human Tubal Fluid (HTF, Irvine Scientific) supplemented with 10% Synthetic Serum Substitute (Irvine Scientific). Epididymal mouse sperm were isolated from retired male breeders and capacitated by incubation with either HTF or Eagle's Minimum Essential Medium (MEM) supplemented with 5% BSA for 1 hour at 37°C (Ho et al., 1995). To assay sperm binding, unfertilized eggs (from normal, from TgN(HuZP3) transgenic, and from Zp<sup>3m/m</sup> (TgN(HuZP3) rescue mice) and normal two-cell mouse embryos were incubated with 500,000/ml motile, capacitated mouse or human sperm in modified MEM (20 μl) under mineral oil in 5% CO<sub>2</sub>/5% O<sub>2</sub>/90% N<sub>2</sub> for 30 minutes. The eggs were washed, to remove non-adherent sperm with a 0.009 inch pipette until the two-cell mouse embryos possessed two to three sperm/egg, then fixed for 2 hours in 1% paraformaldehyde/2% PVP in PBS, and mounted to quantify adherent sperm by difference interference contrast microscopy (Bleil and Wassarman, 1980b).

RESULTS

Establishment of transgenic mouse lines

The 16 kb human ZP3 transgene consisted of 6 kb of the mouse Zp3 promoter ligated to the 5′ half of human ZP3 (exons/introns 1-4) followed by intron-less cDNA (exons 5-8) and a bovine growth hormone (BGH) polyadenylation signal (Fig. 1A). After pronuclear injection and transfer of embryos to 6 foster mothers, the transgene was detected in three of 26 offspring. These animals (2 females, 1 male) were used to establish human ZP3 transgenic mouse lines [TgN(HuZP3);NIH, TgN(HuZP3)2NIH, TgN(HuZP3)3NIH] that have been stably maintained for more than 3 years.

The presence of the human ZP3 transgene was confirmed by Southern blot analysis of genomic DNA digested with two restriction enzymes (BglII, BamHI) and probed with a <sup>32</sup>P-labeled DNA fragment from the common promoter region. In each of the three lines, the expected restriction fragments representing the human ZP3 transgene and the endogenous mouse Zp3 gene were detected (Fig. 1B). Only the endogenous mouse Zp3 restriction fragments were detected in normal littermates. By comparing the intensity of the transgenic promoter band with that of the single-copy endogenous Zp<sup>3</sup> band (Chamberlin and Dean, 1989; Lunsford et al., 1990), we have estimated that the TgN(HuZP3)1NIH line has 4-6 copies and the TgN(HuZP3)2NIH and TgN(HuZP3)3NIH lines each have 2 copies of the human ZP3 transgene.

Oocyte-specific expression of the human ZP3 transgene

Human ZP3 transcripts (1.8 kb) were detected in ovarian RNA by northern blot analysis in each of the three mouse lines (data...
Ovary-restricted expression was confirmed using a sensitive RNase protection assay that distinguished between the human and mouse ZP3 transcripts (Fig. 1C). For each transgenic line, total RNA was obtained from liver, spleen, brain, kidney, stomach, muscle, heart, thymus, lung, uterus, ovary and testis. Human ZP3 transcripts were detected only in the ovary (Fig. 1C, lane 15). Mouse ZP1, ZP2 and ZP3 transcripts were present in ovaries isolated from transgenic lines in amounts comparable to that observed in the normal mouse, suggesting that the expression of the human ZP3 transgene did not adversely affect expression of the endogenous mouse zona genes (data not shown).

To further localize zona gene expression to a specific cell type, ovaries from 3-week old mice were fixed, serially sectioned and hybridized in situ with antisense 35 S-labeled RNA probes specific to human and mouse ZP3 (Fig. 2). Human ZP3 transcripts were readily detected in the cytoplasm of growing oocytes from transgenic (Fig. 2B) but not normal (Fig. 2A) animals. Both human (Fig. 2B) and mouse (Fig. 2D) ZP3 transcripts were detected in the same oocytes indicating co-expression of the endogenous mouse Zp3 and the transgenic human ZP3 genes. The amount of human and mouse ZP3 transcripts within transgenic oocytes appears qualitatively similar (Fig. 2B, D) and comparable to the amount of mouse ZP3 transcript in normal oocytes (Fig. 2C). Neither mouse or human ZP3 transcripts were detected in resting oocytes or in somatic ovarian cells as previously reported for mouse ZP3 (Epifano et al., 1995b). Mouse and human sense probes gave only background hybridization (data not shown), comparable to the background observed when the human ZP3 anti-sense probe was hybridized to normal mouse ovary (Fig. 2A).

Posttranslational modification of human ZP3 expressed in mouse oocytes

The zona pellucidae from transgenic and normal females were analyzed by western blots using monoclonal antibodies specific to mouse or human ZP3. Human ZP3 was detected as a 64 kDa (average) protein that co-migrated with ZP3 from transgenic and normal females. Human ZP3 was detected as a 64 kDa (average) protein that co-migrated with ZP3 from transgenic and normal females.
Human ZP3 rescues infertility of Zp3 null mice

non-viable human eggs (Fig. 3A). The specificity of the monoclonal antibody to an epitope near the carboxyl terminus of human ZP3 indicated expression of full-length recombinant protein. Thus, the apparent molecular mass of human ZP3 expressed in the transgenic mice was indistinguishable from native human ZP3 on these gels (Fig. 3A), but distinctly different from the endogenous mouse ZP3 (83 kDa). These data suggest that the human ZP3 protein expressed in mouse eggs was post-translationally modified as the native human ZP3, although these analyses did not preclude minor differences between native and transgenic human ZP3.

The amount of human ZP3 and of mouse ZP3 detected in huZP3 transgenic mice was less than that observed in native human and mouse zonae, respectively (Fig. 3A). This is consistent with mouse ZP3 and human ZP3 competing for the same sites within a zona matrix having a fixed stoichiometry of ZP1, ZP2 and ZP3 proteins. Additionally, native human zonae pellucidae are considerable thicker than mouse zonae, and the greater intensity of native human ZP3 compared to human ZP3 expressed in mouse oocytes (Fig. 3A, lanes 1,3) may, in part, reflects this increased protein content.

Although human and mouse ZP3 were readily detected in the huZP3 transgenic zona pellucida by antibodies (Fig. 3A), potential differences in antibody affinity complicated using immunoreactivity as a criterion for their relative abundance. Therefore, zonae pellucidae from normal, and huZP3 transgenic female mice were purified by Percoll gradient and analyzed by silver-stained SDS-PAGE (Fig. 3B). The amounts and migration of mouse ZP1 and ZP2 were comparable in both mouse lines. The ZP3 band in huZP3 transgenic zonae, composed of mouse and human ZP3 protein, (Fig. 3B, lane 2) migrated as a broader band (average 78 kDa) than did mouse ZP3 alone (Fig. 3B, lane 1 alone (average 83 kDa). Densitometry scans were unable to resolve the mouse and human ZP3 proteins in the chimeric zonae, and while human ZP3 was present throughout the zona matrix (see below), there appeared to be less human than mouse ZP3 in transgenic zonae pellucidae. Therefore, to remove mouse ZP3 from the chimeric zona matrix, transgenic mice expressing human ZP3 were crossed with Zp3tm/tm null mice to obtain huZP3 rescue mice that expressed only human ZP3.

**Ovarian histology of huZP3 transgenic and huZP3 rescue mice**

These rescue mice were analyzed to ascertain if human ZP3 was sufficiently similar to mouse ZP3 to reconstitute a chimeric zona pellucida and rescue the infertile Zp3tm/tm phenotype. The ovaries of the female huZP3 rescue mice appeared grossly normal and follicles of all developmental stages were present, including corpora lutea indicative of past ovulations. Most notably, the expression of the human ZP3 transgene in a Zp3tm/tm null background resulted in the formation of a zona pellucida matrix around developing oocytes (Fig. 4A), whereas there were no zonae pellucidae present in the ovaries isolated from Zp2tm/tm null mice (Fig. 4B). The reconstituted zona matrix in huZP3 rescue mice appears somewhat thinner in cross-section than that present in
normal litter mates (Fig. 4C) and in TgN(HuZP3) transgenic females (Fig. 4D).

Using monoclonal antibodies specific to each of the zona proteins, ovarian sections of normal and huZP3 rescue mice were examined immunohistochemically. Mouse ZP1, ZP2 and ZP3 (but not human ZP3) were detected in normal mice (Fig. 5A,C,E,G) whereas, mouse ZP1, mouse ZP2 and human ZP3 (but not mouse ZP3) were present in huZP3 rescue zonae pellucidae (Fig. 5B,D,F,H). If the stoichiometry of the three zona proteins is preserved, the thinner zona pellucida surrounding huZP3 rescue oocytes most likely reflects reduced human ZP3 expression, although structural differences between mouse and human ZP3 could also interfere with efficient matrix formation.

Fertility and sperm binding

To assay the fertility of mice expressing human ZP3, five huZP3 transgenic, five huZP3 rescue and five normal females were mated with males, proven to be fertile. On average, the litter sizes of normal (7.4±1.8) and huZP3 transgenic (7.1±2.7) mice were statistically equivalent. HuZP3 rescue females also gave birth to normal pups, but had average litter sizes that were smaller (4.7±0.5) than normal females, albeit the differences were minor (Table 1) and have not be observed in later breeding studies. Following gonadotrophin stimulation, a similar number of ovulated eggs were isolated from huZP3 rescue (21.3±3.5) as from normal (24±4.3) females (Table 1). In distinct contrast, few if any eggs were recovered from in Zp3tm/mtm null females (Liu et al., 1996; Rankin et al., 1996).

Thus, it appears that the thinner zona surrounding the huZP3 rescue eggs does not perturb oocyte growth or folliculogenesis as reflected in a normal complement of ovulated eggs. However, the thinner zona may affect passage down the oviduct causing pre-implantation embryonic loss and resulting in slightly smaller litters. This possibility, however, remains to be quantitatively demonstrated in huZP3 rescue females.

There are considerable in vitro data supporting ZP3 as the primary sperm receptor in mice (Bleil and Wassarman, 1980b, 1988; Florman and Wassarman, 1985;) and normally, human
sperm will not bind to ovulated mouse eggs (Bedford, 1977). If ZP3 was solely responsible for sperm binding, we anticipated that the genetic replacement of ZP3 from one species with that of another might alter the specificity of sperm binding. Ovulated eggs from normal, huZP3 transgenic and huZP3 rescue mice were freed from their cumulus masses and divided into 2 aliquots. One was assayed for mouse sperm binding and the other for human sperm binding. As previously reported, capacitated mouse sperm bound avidly to normal eggs (17.7±2.5 s.e.m./egg, n=54), whereas human sperm did not bind at all (Fig. 6A,B). The ability of mouse sperm to bind to huZP3 transgenic eggs (15.7±5.2 sperm/egg, n=10) assured the functional integrity of their zona pellucidae (Fig. 6C). However, despite the presence of human ZP3 in the zona pellucida, human sperm did not bind to huZP3 transgenic eggs (Fig. 6D) nor to the huZP3 rescue eggs (Fig. 6F), a result that was obvious even prior to washing with semen from three different donors. Although aliquots from the same human sperm samples were successfully used for human in vitro fertilization, there was no increased concentration of human sperm at the surface of the eggs obtained from the huZP3 transgenic or huZP3 rescue mice and no evidence of even transient sperm attachment to the chimeric human-mouse zona pellucida. Similar results were obtained with huZP3 rescue animals derived from two independently established huZP3 transgenic lines and resultant huZP3 rescue lines.

We further observed that despite the absence of mouse ZP3 in the zona pellucida, mouse sperm still bound to huZP3 rescue eggs (17.6±1.8 s.e.m./egg, n=38) (Fig. 6E), even after washing with a pipette to remove all but 1-2 sperm from control 2-cell mouse embryos (to which sperm will not bind physiologically). The in vitro binding of mouse sperm to the huZP3 rescue eggs was correlative with the observed in vivo fertility of huZP3 rescue females (Table 1). Thus, although the presence of human ZP3 in the transgenic zona pellucida was insufficient to support human sperm binding, it did not affect either in vitro mouse sperm binding or in vivo mouse fertility.

**DISCUSSION**

Mice lacking endogenous ZP3 do not form a zona pellucida and are infertile (Liu et al., 1996; Rankin et al., 1996). We have reversed this infertile phenotype by crossing transgenic mice expressing human ZP3 into mouse Zp3 null mice. HuZP3 rescue females reconstitute a chimeric zona pellucida that is composed of mouse ZP1, mouse ZP2 and human ZP3. Although the proteins that compose the zona pellucida are well conserved among mammals and homologues are present in other vertebrates (Lyons et al., 1993; Murata et al., 1995; Chang et al., 1996, 1997; Murata et al., 1997; Hedrick, 1996; Tian et al., 1997; Yang and Hedrick, 1997; Kubo et al., 1997), sperm binding is relatively specific among mammalian orders and human sperm will not bind to mouse eggs. Mouse and human ZP3 share 67% identical amino acids (among 424), but are posttranslationally modified to 83 kDa and 64 kDa, respectively. Human ZP3 expressed in transgenic mice has a molecular mass indistinguishable from native human ZP3 and distinct from mouse ZP3. Nevertheless, despite the presence of human ZP3, human sperm do not bind to huZP3 rescue eggs in vitro and, notwithstanding the absence of mouse ZP3, mouse sperm still bind in vitro and the huZP3 rescue females are fertile in vivo.

In the prevailing paradigm for mouse fertilization, acrosome-intact sperm penetrate the cumulus oophorus surrounding ovulated eggs within the oviduct (Storey et al., 1984). The subsequent attachment of sperm to the zona matrix induces the acrosome reaction in the bound sperm; sperm that have spontaneously undergone the acrosome reaction prior to zona binding do not attach to the zona matrix (Saling et al., 1979; Florman and Storey, 1982). After penetration of the zona

---

**Table 1. Number of super-ovulated eggs and offspring**

<table>
<thead>
<tr>
<th>Eggs/animal</th>
<th>Pups/litter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (Zp3tm/tm)</td>
<td>24±4.3 (14)</td>
</tr>
<tr>
<td>Transgenic [TgN(HuZP3)]</td>
<td>7.4±1.8 (26)</td>
</tr>
<tr>
<td>Rescue [Zp3tm/tm, TgN(HuZP3)]</td>
<td>7.1±2.7 (5)</td>
</tr>
</tbody>
</table>

1 Mean ± s.e.m. (number of animals).

2 Mean ± s.e.m. (number of litters); using Student's t-test with P=0.02, there was no significant difference between normal and rescue litter sizes.
pellucida, only acrosome-reacted sperm bind and fuse to the plasma membrane (Wolf and Hamada, 1979). Thus, the physiologically relevant induction of the acrosome reaction appears to take place at the surface of the zona during initial sperm binding. We observe that normal, huZP3 transgenic and huZP3 rescue mice are fertile in vivo and that mouse sperm bind in vitro to ovulated eggs isolated from each group of animals. Presumably, the sequence of events outlined above that occurs with normal eggs also occurs with eggs isolated from huZP3 transgenic and huZP3 rescue females. Thus, the presence of human ZP3 within the zona matrix does not perturb normal zona pellucida function in mouse fertilization.

There is considerable in vitro evidence that ZP3 plays a primary role in initial sperm-egg binding in mice. After isolation by preparative SDS-PAGE and renaturation, mouse ZP3 (but not ZP1 or ZP2) inhibits sperm-binding to ovulated mouse eggs in vitro (Bleil and Wassarman, 1980b) and this inhibitory activity has been ascribed to a class of O-linked oligosaccharide side chains (Florman and Wassarman, 1985). In earlier studies using a similar mouse Zp3 promoter, hamster ZP3 (422 amino acids, 81% identical to mouse ZP3) expressed in transgenic mice was posttranslationally modified to a molecular mass (56 kDa) identical to native hamster protein and incorporated into a hamster-mouse zona matrix. Using mouse gametes, hamster ZP3 isolated from transgenic mice was judged biologically active by its ability to inhibit sperm binding to eggs, to induce the acrosome reaction, and to participate in a postfertilization block to polyspermy (Kinloch et al., 1992). Based on these observations, it was anticipated that if ZP3 is a primary sperm receptor in humans, then heterologous human ZP3 expressed in transgenic mice would comparable to native human ZP3 on SDS-PAGE, human sperm do not bind to the chimeric zona pellucida in vitro. Within the paradigm that sperm binding induces the acrosome reaction, the absence of even transient binding indicates that the downstream events necessary for in vitro fertilization do not occur. However, the success of in vitro human fertilization with the same semen sample demonstrates that the lack of binding to the chimeric zona pellucida is not intrinsic to the human sperm.

There appear to be several possible explanations for the unanticipated persistence of order-specific binding after replacement of mouse ZP3 with human ZP3 in chimeric zonae pellucidae. Firstly, posttranslational modifications of human ZP3 expressed in the rescue mouse oocyte could provide mouse-specific biological activity. Mouse spermatozoa have been reported to recognize and bind in vitro to a distinct class of oligosaccharides (Florman and Wassarman, 1985), the bioactivity of which has been variously ascribed to a terminal α1,3 galactose (Bleil and Wassarman, 1988) or N-acetylgalactosamine residues (Miller et al., 1992). The continued fertility of mice lacking α1,3- (Thall et al., 1995) or B1,4-galactosyl transferase (Lu and Shur, 1997), however, suggests additional complexity to sperm binding. Although human ZP3 from the transgenic mice co-migrates on SDS-PAGE with native human ZP3 (64 kDa) and is distinct from native mouse ZP3 (83 kDa), it may be that minor carbohydrate modifications in the huZP3 rescue oocytes are sufficient to impart mouse-specific sperm binding to human ZP3 while precluding human sperm binding. There is precedence for differential glycosylation in the same cell type isolated from mammals and mouse-specific, posttranslational modifications of ZP3 would be consistent with prevailing paradigms for mouse fertilization. Secondly, the supramolecular structure of the zona pellucida matrix could be a major determinant of sperm binding specificity. Although the mouse and human ZP3 proteins are well conserved (77% amino acid similarity; 12 identical cysteine residues), it may be that within a three-dimensional zona matrix containing mouse ZP1 and ZP2, human ZP3 is not arrayed correctly to permit human sperm recognition, but is similar enough to mouse ZP3 to permit mouse sperm binding. Thirdly, another of the three zona protein(s) may be the critical component for species-specific sperm binding. For example, there is evidence that ZPB and/rc55 (ZP1 homologues) mediate sperm binding in pigs and rabbits, respectively, (Prasad et al., 1996; Sacco et al., 1989; Yonezawa et al., 1995) and that gp64/69 (a ZP2 homologue) mediates sperm binding in frogs (Tian et al., 1997). Thus, the primary adhesion of mouse and human sperm binding could involve ZP1 or ZP2 by themselves or in combination with one or more other zona proteins as has been suggested for pig sperm binding (Yurewicz et al., 1998).

Although ZP3 has been evolutionarily conserved for 650 million years as a component of extracellular matrices surrounding vertebrate eggs, it is not always involved in sperm binding. Teleostean fish have a vitelline envelope that morphologically resembles the zona pellucida and is composed of two proteins, one of which has a similar length (420-424 amino acids) and is 36-43% identical to mouse ZP3 (Murata et al., 1995; Chang et al., 1996). However, teleostean fish sperm lack an acrosome and neither bind nor physically penetrate the vitelline envelope. Rather, fish sperm are attracted to the outer opening of a micropyle, a funnel-shaped tunnel crossing the vitelline envelope that is sufficiently narrowed at the inner opening to allow only one sperm to fuse with and fertilize the egg (Yamagami et al., 1992; Amanze and Iyengar, 1990). The carboxyl half of the second teleostean vitelline envelope protein (Lyons et al., 1993; Chang et al., 1997; Murata et al., 1997) is similar to mouse ZP1 and ZP2 which share a conserved carboxyl terminal domain (Schwoebel et al., 1991). In the winter flounder this domain is encoded by 6 exons and is 35% identical to 250 amino acids of mouse ZP1 and ZP2 (Lyons et al., 1993; Epifano et al., 1995a). Thus, it appears that two primitive genes encoded proteins for a vitelline envelope. One, the ZP3 homologue, is well conserved between fish and mammals. The other, has undergone partial duplication and more evolutionary divergence. Whether the newer biological functions of human sperm binding and induction of the sperm acrosome reaction are attributable to these more modern domains in the human zona proteins remain to be determined.

We thank Drs S. Daniel and F. Chang of the Center for Human Reproduction - MidAtlantic for their assistance in the procurement of clinical specimens and appreciate the critical reading of the manuscript by Drs H. Florman and M. O’Rand.

REFERENCES


Lu, Q. and Shur, B. D. (1997). Sperm from B1,4-galactosyltransferase-null mice are refractory to ZP3-induced acrosome reactions and penetrate the zona pellucida poorly. Development 124, 4112-4113.


Schwoebel, E., Prasad, S., Timmons, T. M., Cook, R., Kimura, H., Niu, E.,


