A single domain of the ZP2 zona pellucida protein mediates gamete recognition in mice and humans

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Introduction

Monospermic fertilization is required for successful development in mice and humans. However, the molecular basis of sperm–egg interaction remains incompletely understood despite decades of investigation. Ovulated eggs are surrounded by an extracellular zona pellucida, to which sperm bind and penetrate before gamete fusion. After fertilization, the zona matrix is modified so that sperm do not bind to the early embryo. Humans have four genetic loci encoding ZP1, ZP2, ZP3, and ZP4 (Spargo and Hope, 2003), but mouse Zp4 is a pseudogene (Lefèvre et al., 2004), and the mouse zona pellucida contains only three glycoproteins (Bleil and Wassarman, 1980). Given the simple structure of the zona pellucida, it has been surprisingly difficult to genetically define a zona protein that is essential for fertilization and the postfertilization block to polyspermy.

To identify the zona ligand for sperm recognition, we have exploited two physiological dichotomies. One is that sperm bind to the zona pellucida surrounding eggs before, but not after, fertilization. The only biochemically documented change in the zona matrix is the postfertilization cleavage of ZP2 (Bleil et al., 1981; Bauskin et al., 1999), and the site has been defined as LA↓DE in mice (Gahlay et al., 2010). If mutated in transgenic mice or if the cleaving enzyme, ovastacin, is genetically ablated, ZP2 remains intact and sperm bind de novo to the zona pellucida surrounding early embryos despite fertilization (Gahlay et al., 2010; Burkart et al., 2012). The second dichotomy is that human sperm bind to human, but not mouse, zonae pellucidae (Bedford, 1977).

To capitalize on these differences, we have established transgenic mouse lines lacking ZP2 and expressing truncated or human/mouse chimeric isoforms to define ZP251–149 as the zona ligand for human and mouse sperm recognition, and document that in its absence female mice are sterile.

Results and discussion

Female mice that form a zona pellucida lacking ZP2 are sterile

Formation of the extracellular zona matrix is mediated by zona domains (260 aa motifs with conserved cysteine residues) present near the C terminus of each secreted zona protein (Bork and Sander, 1992). The human zona pellucida is composed of four (ZP1–4) and the mouse of three (ZP1–3) glycoproteins (Bleil and Wassarman, 1980; Bauskin et al., 1999). Mouse ZP1, the least

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Figure 1. A zona pellucida is formed with ZP4 in the absence of ZP2, and female mice are sterile. [A] Phylogeny of mouse and human zona proteins indicates two clades; one composed of ZP1, ZP2, and ZP4 and the other of ZP3. There is no mouse ZP4 protein because of multiple stop and missense codons in the cognate gene. Mya, million years ago. (B) Schematic representation of the four zona pellucida proteins with 8 or 10 conserved cysteine residues. The resultant disulfide bonds differ in the zona domains of the ZP1/2/4 and ZP3 clades and are indicated as A and B, respectively. The postfertilization cleavage site is marked on ZP2, and both ZP1 and ZP4 contain trefoil domains. (C) Glutaraldehyde-fixed, plastic-embedded ovarian sections (3 µm) from 8–10-wk-old normal, Zp2<sup>−/−</sup>, moQuad<sup>huZP4</sup>, and moQuad-Zp2<sup>−/−</sup> mice were merged and faux colored. (D) Formaldehyde-fixed moQuad-Zp2<sup>−/−</sup> eggs stained with protein-specific monoclonal antibodies. Fluorescent and DIC images were merged and faux colored. [E] Mouse sperm binding to moQuad<sup>huZP4</sup> and moQuad-Zp2<sup>−/−</sup> eggs. Inset, 2.5x magnification. Zp3<sup>EGFP</sup> mouse eggs (green zona) and mouse two-cell embryos were positive and negative sperm-binding controls, respectively. Schematics to the left reflect protein composition of the zonae pellucidae with the source of sperm below.

abundant protein, is not required for formation of the zona pellucida or fertility (Rankin et al., 1999). In the absence of ZP2, a thin zona matrix is formed around growing oocytes that does not persist in ovulated eggs (Rankin et al., 2001), and no zona matrix is formed in the absence of ZP3 (Liu et al., 1996; Rankin et al., 1996). Based on the phylogeny (Larkin et al., 2007) of human and mouse zona proteins, ZP1/ZP2/ZP4 fall into one clade and ZP3 into another (Fig. 1 A). The zona domains of ZP2 and ZP3 have 10 and 8 conserved cysteine residues, respectively, the linkage of which differs in the two clades (Fig. 1 B; Boja et al., 2003). We reasoned that if members of the first clade could substitute for one another, the presence of ZP4 along with ZP1 might permit formation of a matrix in the absence of mouse ZP2 and provide a loss-of-function assay for sperm–egg recognition. Because mouse Zp4 is a pseudogene (Lefèvre et al., 2004), human ZP4 was expressed in transgenic mice (Yauger et al., 2011) to establish a mouse line designated moQuad (moZP1, moZP2, moZP3, and huZP4). After the appropriate crosses, mice lacking mouse ZP2 in the presence of ZP4 were established and designated moQuad-Zp2<sup>−/−</sup> (moZP1, moZP3, and huZP4; Table S1).

In the presence of huZP4, moQuad<sup>huZP4</sup> (ZP1,2,3,4) and moQuad-Zp2<sup>−/−</sup> (ZP1,3,4) transgenic lines form zona pellucidae during oocyte growth that is similar to normal oocytes (Fig. 1 C and Table S1). The zona pellucida persists after eggs are ovulated into the oviduct, and the composition of the moQuad-Zp2<sup>−/−</sup> zona matrix was confirmed with monoclonal antibodies that documented the absence of mouse ZP2 (Fig. 1 D). Ovulated eggs in cumulus (hyaluron interspersed with follicular cells) from moQuad<sup>huZP4</sup> and moQuad-Zp2<sup>−/−</sup> mice were inseminated with mouse sperm using Zp3<sup>EGFP</sup> mouse eggs (green zona) and normal mouse two-cell embryos, respectively, as positive and negative controls. Mouse sperm bound to moQuad<sup>huZP4</sup> (41.4 ± 2.5, n = 30), but not to moQuad-Zp2<sup>−/−</sup>, eggs (1.6 ± 0.3, n = 43; Fig. 1 E), and the latter, but not the former, mouse line was sterile (Table S2). Thus, mouse ZP2 is required for in vitro sperm binding and in vivo mouse fertility.

Truncated ZP2 does not support mouse sperm binding
The secreted ZP2 ectodomain (35–633 aa) lacks the signal peptide (1–34) that directs ZP2 into the endosomal pathway and the C terminus (634–713) that includes a transmembrane domain. After fertilization, the ectodomain is cleaved near the N terminus (Δ<sup>166</sup>LA<sub>149</sub>DE<sup>169</sup>) by ovastacin, an egg cortical granule metalloendoprotease, after which sperm no longer bind to the zona pellucida (Gahlay et al., 2010; Burkart et al., 2012). To investigate the importance of the N terminus in mouse gamete recognition, DNA recombineering was used to construct a transgene lacking Zp2<sup>166–149</sup> (moZp2<sup>166–149</sup>; Fig. 2 A and Fig. S1 A). After crossing into the Zp2<sup>−/−</sup> background, female mice reconstituted a zona pellucida that was thinner than normal. Therefore, these lines were crossed with huZP4 transgenic mice to establish moQuad-Zp2<sup>166–149</sup> mice that developed a more robust zona matrix during oocyte growth (Fig. 2 B).

The zona pellucida surrounding ovulated eggs from moQuad and moQuad-Zp2<sup>166–149</sup> mice were analyzed by confocal microscopy using ZP2 domain-specific monoclonal antibodies. The zonae from both genotypes reacted with antibodies to ZP1, ZP2<sup>C-term</sup>, ZP3, and huZP4. However, the monoclonal antibody...
to the N terminus of ZP2 did not react with the zona pellucida surrounding moQuad-Zp2\textsuperscript{Trunc} eggs, although it recognized normal ZP2 in the moQuad (Fig. 2 C). On immunoblots, the monoclonal antibody to the ZP2\textsuperscript{N-term} detected a 120-kD band in the zona pellucida isolated from moQuad, but not moQuad-Zp2\textsuperscript{Trunc} eggs (Fig. 2 D, left). A monoclonal antibody to the ZP2\textsuperscript{C-term} detected a 120-kD band in the zona pellucida isolated from moQuad eggs and a 92-kD band in eggs from moQuad-Zp2\textsuperscript{Trunc} mice (Fig. 2 D, right). These observations are consistent with deletion of 99 amino acids (ZP2\textsuperscript{51–149}) in the N terminus of ZP2 of the zona pellucida surrounding moQuad-Zp2\textsuperscript{Trunc} eggs.

Mouse sperm did not bind to either Zp2\textsuperscript{Trunc} (0.6 ± 0.2, n = 22) or moQuad-Zp2\textsuperscript{Trunc} (2.3 ± 1.3, n = 30) eggs (Fig. 2 E), and, when mated with normal males, moQuad-Zp2\textsuperscript{Trunc} females were sterile (Table S2). Based on these observations in transgenic mice, we conclude that the N terminus of mouse ZP2 is necessary for gamete recognition in vitro and in vivo.

**Human sperm recognize human but not mouse ZP2**

To extend these observations to human biology, huQuad\textsuperscript{(huZP1–4)} mice containing all four human proteins and none of the endogenous mouse proteins (Baibakov et al., 2012) were used to establish huQuad-ZP2\textsuperscript{Null} (huZP1,3,4) mouse lines (Table S1). Both lines formed a zona surrounding growing oocytes within the ovary (Fig. 3 A), and, using monoclonal antibodies specific to the human proteins, the absence of huZP2 in huQuad-ZP2\textsuperscript{Null} ovulated eggs was confirmed (Fig. 3 B). After insemination with human semen, sperm bound robustly to huQuad\textsuperscript{(huZP1–4)} eggs (59.8 ± 5.3, n = 37), but rarely to huQuad-ZP2\textsuperscript{Null} eggs (1.5 ± 0.3, n = 36; Fig. 3 C).

To ascertain if mouse ZP2 exerted a non-taxon-specific effect on the zona structure important for gamete recognition, mouse Zp2 was expressed in huQuad-ZP2\textsuperscript{Null} eggs. However, human sperm did not bind to the zona pellucida surrounding huQuad-ZP2\textsuperscript{Null}, Zp2\textsuperscript{M} eggs (0.7 ± 0.2, n = 30; Fig. 3 C).

To determine if these observations pertain in vivo, an artificial insemination assay was established using mouse sperm as a positive control for fertility and for accumulation of sperm in the perivitelline space. After transcervical insemination (Fig. S2 A), control \((Cd^{9+/+})\) mice were fertile, albeit with smaller litters \((2.0 ± 0.54 vs. 10.4 ± 0.81 for natural mating). \(Cd^{9+/+}\) eggs to which sperm will not fuse \((Le Naour et al., 2000)\) accumulated 1–6 mouse sperm in their perivitelline spaces \((between the inner aspects of the zona matrix and the plasma membrane)\) in 9 of 113 eggs \((Fig. 3 D). Subsequently, huZP2\textsuperscript{Rescue} (huZP2 replaces moZP2) and huZP3\textsuperscript{Rescue} \((huZP3 replaces moZP3) mice \((Table S1)\) were inseminated with human sperm \((3 × 10^7)\) and eggs recovered from the oviduct were examined by confocal microscopy to detect sperm in the perivitelline space where they accumulated, unable to fuse with mouse eggs. Of 29 eggs recovered from four huZP2\textsuperscript{Rescue} mice, human sperm were detected in the perivitelline space of four eggs \((one egg per female), which is consistent with the small litters observed in control \((Cd^{9+/+})\) female mice. No sperm were observed in the perivitelline space of 28 huZP3\textsuperscript{Rescue} eggs \((Fig. 3 E). To facilitate access to ovulated eggs, human sperm also were transferred directly to the oviduct \((Fig. 3 F). After 2 h of incubation in vivo, 1–5 human sperm were observed in the perivitelline space of 22–77 eggs \((29%)\) from huZP2\textsuperscript{Rescue} mice \((Fig. 1 G). No sperm were observed in the perivitelline space of 85 control huZP3\textsuperscript{Rescue} eggs, which is consistent with the small litters observed in control \((Cd^{9+/+})\) female mice.
consistent with their inability to support human sperm binding in vitro (Baibakov et al., 2012). Thus, human sperm bind and penetrate the zona pellucida of huQuad-ZP2Rescue, but not huQuad-ZP3Rescue, eggs in vitro and in vivo.

Mouse sperm, lacking taxon-specific gamete recognition (Bedford, 1977), also bound to huQuad expressing human ZP2 (35.3 ± 1.9, n = 52) and the huQuad-ZP2Null, Zp2Mo (36.3 ± 2.2, n = 36) expressing mouse ZP2, but not the huQuad-ZP2Null eggs missing both mouse and human ZP2 (2.4 ± 0.3, n = 44; Fig. S2 B). After mating, huQuad(huZP1–4) and huQuad-ZP2Null female mice containing human or mouse ZP2 in their zonae pellucidae were fertile, but huQuad-ZP2Null female

Figure 3. Human sperm binding to the zona pellucida requires human ZP2. (A) Ovarian histology from huQuad(huZP1–4) and huQuad-ZP2Null mice as in Fig. 1 C. (B) Eggs from huQuad-ZP2Null stained with monoclonal antibodies as in Fig. 1 D. (C) Human sperm binding to huQuad(huZP1–4), huQuad-ZP2Null, and huQuad-ZP2Null, Zp2Mo eggs (as in Fig. 1 E) using noninseminated human oocytes and mouse Zp3EGFP eggs (green zona) as positive and negative controls, respectively. (D) Litter sizes after transcervical insemination of control (Cd9−/−) mice compared with natural mating (top). Sperm in the perivitelline space (PVS) of Cd9−/− eggs after transcervical insemination with mouse sperm (bottom). Recovered eggs (left) and the number of sperm in PVS (right). Arrows indicate sperm. (E) As in D (bottom) but with huZP2Rescue (top) and huZP3Rescue (bottom) eggs after transcervical insemination with human sperm. (F) In vivo oviduct transfer of human sperm (2.3 × 10^3 sperm in 0.5 µl) to hormonally stimulated, anesthetized huZP2Rescue and huZP3Rescue female mice. (G) As in E, but after in vivo oviductal transfer. (H) Normal mouse, huZP2Rescue, huQuad eggs, and human oocyte stained with antibody to the sialyl-Lewis X antigen.
A ZP2 domain mediates gamete recognition

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The ZP2 domain required for sperm binding regulates taxon-specific gamete recognition

The ectodomains of human (602 aa) and mouse (599 aa) ZP2 share 62% amino acid identity, but the N termini of the two ZP2 proteins are only 48% identical. Thus, we questioned whether the taxon specificity is mediated by the gamete recognition domain found to be essential for normal mouse sperm binding and fertility in Zp2Trunc mice. Using DNA recombineering, genomic regions encoding the N termini of mouse and human ZP2 were replaced with the corresponding human exons encoding human ZP222–164 and mouse exons encoding mouse ZP218–156, respectively (Fig. S1A). After establishment by pronuclear injection, each transgenic line was crossed into the mouse Zp2Null line to eliminate endogenous ZP2 protein and designated hu/moZP2 Rescue and mo/huZP2 Rescue mice, respectively (Fig. 4A). The mice appeared normal and a robust zona pellucida was observed in ovarian sections from hu/moZP2 Rescue and mo/huZP2 Rescue female mice (Fig. 4B).

Mouse ZP1 and ZP3 were present in the zona pellucida surrounding ovulated eggs from each of the three transgenic mouse lines lacking mouse and human ZP2 were sterile (Table S2). Collectively, these results indicate that ZP2 is necessary for human and mouse sperm binding and penetration through the zona pellucida, and for mouse fertility.

Earlier models of gamete recognition had focused on the role of specific glycans as ligands for sperm binding (Florman and Wassarman, 1985; Bleil and Wassarman, 1988; Miller et al., 1992; Chen et al., 1998). However, continued fertility after mutation of attachment sites (Liu et al., 1995; Gahlay et al., 2010) and genetic ablation of specific galactosyltransferases (Thall et al., 1995; Lowe and Marth, 2003; Shi et al., 2004; Williams et al., 2007) have not supported the candidacy of any of the proposed glycans. More recently, the sialyl-Lewisx antigen has been reported to mediate human sperm binding to human zona pellucidae (Pang et al., 2011). Although the sialyl-Lewisx antigen was detected in the zona pellucida surrounding control human oocytes, it was not present in the zona matrix formed by normal, huZP2Rescue, or huQuad(huZP1–4) eggs (Fig. 3H) and cannot account for the observed binding of human sperm under these experimental conditions.

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Perspective
ZP2 was first proposed as a primary sperm-binding ligand in *Xenopus laevis* (Tian et al., 1999) and more recently in humans (Baibakov et al., 2012). However, like all other candidates, it had not been defined as essential by genetic ablation. We now report that transgenic mice expressing human ZP4 form a zona pellucida in the absence of ZP2. Using this model system, we have identified a domain within ZP2 that accounts for the taxon specificity of human sperm binding to the zona pellucida and, by genetic ablation, document that this domain is essential for female mouse fertility. Collectively, these results are consistent with a model (Fig. 5) in which mouse and human sperm bind to ZP2P1–149 and hyperactive sperm penetrate the zona matrix to fuse and fertilize ovulated eggs. This triggers egg cortical granule exocytosis in which ovastacin, a metalloendoprotease (Quesada et al., 2004) encoded by *Astl*, is released and cleaves upstream of a di-acidic motif ([143LA]DE169) in ZP2 (Burkart et al., 2012). Although a single cleavage site is reported in human ZP2 (Bauskin et al., 1999), multiple degradation products are observed in mouse ZP2 (Burkart et al., 2012). In each case, the postfertilization cleavage of ZP2 provides a definitive block to polyspermy. Sperm that do not bind to the zona pellucida cannot penetrate the zona matrix or fuse with the egg plasma membrane. The destruction of the N terminus of ZP2 provides an effective block to polyspermy and ensures the monospermic fertilization required for the successful onset of development.

Materials and methods

Transgenic mouse lines
Targeted mutagenesis with a PGK-neomycin cassette was used to genetically ablate Zp1 (replaced exon 1–3), Zp2 (replaced exon 1–2), Zp3 (replaced exon 1), and Cd9 (replaced promoter and exon 1) in embryonic stem (ES) cells. Correctly targeted ES cells were identified for injection into blastocystcs to establish mouse lines with null alleles (Rankin et al., 1996, 1999, 2001; Le Naour et al., 2000). Cd9 null mice were a gift of C. Boucheix (Institut National de la Sante et de la Recherche Medicale, Villejuif, France). Mouse lines expressing human zona proteins were established after pronuclear injection of genomic DNA encoding human ZP1 (11.9 kb including 2.2 promoter, 8.1 coding region, and 1.5 of 3’ flank), ZP2 (16.8 kb including 2.3 promoter, 14 kb coding region, and 0.5 kb of 3’ flank), ZP3 (16 kb including 6.0 kb promoter, exons 1–4 followed by cDNA encoding exons 5–8 and a BGH polyadenylation signal), and ZP4 (11.6 kb including 6.0 kb promoter, 14 kb coding region, and 0.5 kb of 3’ flank; Rankin et al., 1998, 2003; Yauger et al., 2011; Baibakov et al., 2012). In addition, three new mouse lines were established using chimeric hu/moZp2, chimeric mo/huZp2, and moZp225–58* transgenes (Fig. S2 A) constructed by Glik DNA recombiner- ing (Warming et al., 2005).

To establish the new lines, bacterial artificial chromosome (BAC) DNA (Life Technologies) that include either mouse Zp2 (RP23-6513) or human Zp2 (RP11-1023A8) were transformed into Sw102 bacterial cells containing the λ prophage recombinase system (Liu et al., 2003). For hu/moZp2, human genomic DNA encoding huZp234–168 (bp 26 in exon
2 to bp 21 in exon 6) replaced mouse genomic DNA encoding moZP237–165 (bp 59 in exon 2 to bp 6 in exon 5). To construct this transgene, a PCR fragment (1,331 bp) containing the galK operon flanked by 50 bp homologous to moZP2 gene 5' and 3' of the sequence encoding moZP222–161 protein was amplified (huZP2-GalK primers; Table S3) using NEB Phusion (New England Biolabs, Inc.). After digestion with DpnI and overnight gel purification (0.7% agarose, 15 V, 16 h), the PCR fragment was electroporated into the BAC containing SW102 cells, and recombinants were selected by growth on minimal media with galactose. Using a clone from this first step, the galK cassette was replaced by recombining with a second PCR fragment (5,783 bp) encoding huZP241–161 protein with 100 bp arms homologous to moZP2 on either side (moZP2-huZP2 primers; Table S3). Mutant clones were selected on minimal media with 2-deoxy-galactose and confirmed by DNA sequencing of PCR products using gene specific primers (Table S3) to isolate 5' and 3' junction fragments.

In a similar recombining strategy, DNA encoding huZP222–161 protein was replaced with sequence encoding moZP218–156 using huZP2-GalK and huZP2moZP2 primers (Table S3), and DNA encoding moZP237–149 protein was removed using moZP2-GalK and bridging moZP2 primers (Table S3) to establish the mo/huZP2 and moZP2transgenic, respectively. For the mo/huZP2 transgene, mouse genomic DNA encoding moZP218–156 (bp 1 in exon 2 to bp 150 in exon 5) replaced human genomic DNA encoding huZP222–161 (bp 1 in exon 2 to bp 152 in exon 5), and for the moZP2transgene, DNA encoding moZP237–149 (bp 59 in exon 2 to bp 27 in exon 6) was deleted (Fig. S1 A).

Nott fragment containing the hu/moZP2 (17.9 kb including 2.3 and 0.9 kb of the 5' and 3' flanking regions, respectively), mo/huZP2 (16.8 kb including 1.5 and 1.7 kb of the 5' and 3' flanking regions, respectively), and truncated moZP2transgenes were retrieved from the BAC with p253 (Gahlay et al., 2010), and the fidelity of coding regions was confirmed by DNA sequencing. After gel purification, the transgenes were injected into the male pronucleus of fertilized FVB/N eggs by the Taconic Transgenic Mouse Facility. At least two founders were established for each transgene and crossed into mouse Zp1-3 null and human ZP1-4 transgenic mouse lines.

Expression of transgenes
Expression of normal Zp2, as well as hu/moZP2, mo/huZP2, and moZP2transgenes, was detected by RT-PCR using as gene specific primer sets (Table S5) and total RNA isolated from 8–12 wk old mouse tissue (Fig. S2 B). Gapdh transcripts served as a control for RNA integrity (Baibakov et al., 2012).

Microscopy
Samples were mounted in PBS, and images of eggs, embryos, and beads were obtained with a confocal microscope (LSM 510; Carl Zeiss) using a 63x/1.2 NA water immersion objective lens at room temperature (Baibakov et al., 2007; Yang et al., 2011). LSM 510 images were exported as full-resolution TIF files and processed in Photoshop CS5.5 (Adobe) to adjust brightness and contrast. Additionally, confocal optical sections were projected to a single plane with maximum intensity and combined with DIC images of eggs or peptide beads using LSM image software.

Ovarian histology and immunohistochemistry
Mature oocytes were fixed in glutaraldehyde and embedded in glycol methacrylate before staining with periodic Schiff’s acid and hematoxylin (Yang et al., 2011). Ovulated eggs were fixed in 2% paraformaldehyde before staining with rat or mouse monoclonal antibodies (1:50) specific to: moZP1 (Rankin et al., 1999), N terminus of moZP2 (East and Dean, 1984), C terminus of moZP2 (Rankin et al., 2003), moZP3 (East et al., 1985), huZP1 (Ganguly et al., 2010), huZP2 (Rankin et al., 2003), huZP3 (Rankin et al., 1998), and huZP4 (Bukovsky et al., 2008). Monoclonal antibodies to human ZP1 and ZP4 were a gift from S. Gupta (National Institute of Immunology, New Delhi, India). Primary antibody binding was detected with goat anti-mouse Alexa Fluor 568 (1:100) or goat anti–rat Cy3 (1:100) antibodies (Life Technologies). Monoclonal antibody CSLEX1 (BD) that binds to the sialy-Lewis’ antigen was dilute 1:50 and detected with goat anti–mouse IgM-FITC (1:20; Life Technologies). Images were obtained at room temperature on a microscope (Carl Zeiss; Axiosplan 2) equipped with a 40x/0.75 NA objective lens and a camera (AxioCam 1CC1) and AxioVision software (all from Carl Zeiss).

Assessment of sperm binding and fertility
To assay mouse sperm binding to the zona pellucida surrounding normal and transgenic mouse eggs, sperm were released from cauda epididymides, capacitated in human tubal fluid (HTF; EMD Millipore) supplemented with 0.4% BSA (Sigma-Aldrich) for 40 min (37°C, 90% N2, 5% O2, and 5% CO2), and added to eggs in cumulus and embryos in 100 µl of HTF. 0.4% BSA at a final concentration of 107 ml–1 progressive motile sperm as determined by HTM-VOS (Version 12.3) motility analyzer (Hamilton Thorne; Oakly et al., 2010). ZP2transgenic mouse eggs (green zone) and two-cell embryos served as positive and negative wash controls, respectively. Samples were fixed in 2% paraformaldehyde and stained with Hoechst to identify nuclei. Bound sperm were quantified from z projections obtained by confocal microscopy (Baibakov et al., 2007), and results reflect the mean ± SEM from at least three independently obtained samples each containing 10–50 mouse eggs/embryos.

To assay human sperm binding, individual aliquots (0.5 ml) of liquefied human semen (Genetics & IVF Institute Fairfax Cryobank) were added to an Eppendorf tube (2.0 ml) containing 0.5 ml of 40% of PureSperm (Nidaco) layered over 5.0 ml of 80% PureSperm. After centrifugation (swinging bucket, 20 min × 300 g, 20°C) and removal of the supernatant, sperm were resuspended in the residual buffer and transferred into 1.0 ml HTF. After a second centrifugation (5 min × 300 g), sperm were resuspended in 0.2 ml of HTF/BSA, and 2–4 aliquots were mixed before evaluation by HTM-VOS. Sperm were then diluted in HTF, 0.5% BSA to 107 ml–1 progressive motile sperm. Transgenic eggs in cumulus, normal eggs, ZP3EGFP eggs (Zhao et al., 2002), and noninseminated, immature human oocytes were incubated in droplets (100 µl) of sperm/HTF, 0.5% BSA under mineral oil (BD) in a BT37GP incubator (Planer). Eggs/embryos/oocytes were washed by serial transfer through 500 µl of HTF/BSA and fixed for imaging (Baibakov et al., 2012). For each experiment using human sperm, initial evaluations were conducted using 10 huZP2transgenic eggs as positive controls to test batches of HTF media and quality of human sperm preparations, which varied.

To assess fertility, females (≥5) from each mouse line were singly co-caged with a fertile female (control, NIH Swiss) and mated [2:1] with a male (NIH Swiss) proven to be fertile. Litters were recorded until the NIH Swiss fertile female gave birth to at least three litters or after 5 mo of mating.

In vivo transcervical insemination
Female mice [8–12 wk old] were hormonally stimulated at 12:00 midnight with 5 IU of pregnant mare serum gonadotrophin (PMSG), and hCG was injected intraperitoneally 48 h later. Epididymal mouse sperm from three males was released into 1.5 ml of HTF, 0.4% BSA, and equilibrated for 1–5 h (37°C, 90% N2, 5% O2, and 5% CO2). 500 µl was used to inseminate each of two female mice. Alternatively, commercially obtained human semen (proven to bind and penetrate human ZP2transgenic eggs in vitro) was prepared as described in “Assessment of sperm binding and fertility” and diluted in 500 µl HTF 0.5% BSA that had been equilibrated [≥24 h] at 37°C, 90% N2, 5% O2, and 5% CO2. After capacitation (1.5 h), the contents of two vials of human semen were used to inseminate a single female mouse. At 11:00 p.m. (1 h after KCG injection), females were restrained and inseminated with mouse or human sperm using an NSET device (ParaTechs) connected to a 1-ml syringe, which reached a single uterine horn without anesthesia and surgery (Snell et al., 1944). Females were mated overnight with sterile, vasectomized males, and ~10 h later, eggs were collected from the oviducts of females with copulatory plugs.

In vivo oviduct transfer of sperm
HuZP2transgenic and huZP2transgenic females were stimulated with gonadotrophins and anesthetized. Human sperm were injected into the oviduct, proximal to the swollen ampulla. 2 h later, the mice were euthanized to collect eggs that were fixed and imaged by confocal microscopy (Sato and Kimura, 2001; Takahiro et al., 2012).
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