

# Abnormal zonae pellucidae in mice lacking ZP1 result in early embryonic loss

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## SUMMARY

All vertebrates have an egg shell that surrounds ovulated eggs and plays critical roles in gamete recognition. This extracellular matrix is known as the zona pellucida in eutherian mammals and consists of three glycoproteins, ZP1, ZP2 and ZP3 in the mouse. To investigate the role of ZP1 in fertilization and early development, we have used targeted mutagenesis in embryonic stem cells to create mouse lines (*Zp1<sup>tm/tm</sup>*) lacking ZP1. Although a zona pellucida composed of ZP2 and ZP3 was formed around growing *Zp1<sup>tm/tm</sup>* oocytes, the matrix was more loosely organized than zonae around normal oocytes. In some *Zp1* null follicles, this structural abnormality resulted in ectopic clusters of granulosa cells, lodged between the zona matrix

and the oolemma, that perturbed normal folliculogenesis. Comparable numbers of eggs were ovulated from *Zp1* null females and normal females following hormonal stimulation. However, after mating with males, fewer two-cell embryos were recovered from *Zp1* null females, and their litters were significantly smaller than those produced by normal mice. Therefore, although mouse ZP1 is not essential for sperm binding or fertilization, it is required for the structural integrity of the zona pellucida to minimize precocious hatching and reduced fecundity.

Key words: Zona pellucida, Fertility, *Zp1* null mutant mice, Embryonic loss, Pre-implantation development

## INTRODUCTION

The mammalian zona pellucida is an extracellular matrix that surrounds eggs and early embryos, and is critical for normal fertilization and pre-implantation development. At fertilization, sperm bind tightly to the zona, penetrate the zona matrix, and fuse with the egg's plasma membrane. The mouse zona is composed of three major glycoproteins (ZP1, ZP2, ZP3), and ZP3 has been implicated in binding sperm in vitro via O-linked oligosaccharide side chains, variously described as terminating in  $\alpha$ 1,3 galactose (Bleil and Wassarman, 1988) or *N*-acetylglucosamine (Lu and Shur, 1997). However, transgenic mice in which mouse ZP3 has been replaced with human ZP3 form a chimeric zona matrix to which mouse, but not human, sperm bind, and these transgenic animals are fertile in vivo (Rankin et al., 1998). Additionally, alternative zona proteins are reported to support sperm binding in other species. For example, the *Xenopus* homologue of ZP2 (gp69/64) inhibits sperm binding in vitro (Tian et al., 1997a), and homologues of ZP1 (by themselves or in combination with ZP3) are reported to be involved in sperm binding in the pig and the rabbit (Prasad et al., 1996; Sacco et al., 1989; Yamasaki et al., 1995; Yurewicz et al., 1998).

After fertilization, the zona pellucida is modified such that no new sperm bind, and those that are bound can no longer penetrate the matrix. This block to polyspermy is associated with modifications of the zona proteins by glycosidases released from cortical granules (Miller et al., 1993) and proteolytic cleavage of ZP2 or its homologue gp69/64, in mice

and frogs respectively (Bleil et al., 1981; Tian et al., 1997b). Whether other zona proteins are biochemically modified on a more subtle basis has been inferred but not yet biochemically demonstrated. The one-cell zygote and early embryo remain surrounded by the zona during oviductal transport until the blastocyst stage, at which time the embryo escapes from the matrix and implants in the wall of the uterus. In genetically altered mice lacking a zona pellucida due to loss of ZP3, 2-cell embryos are not recovered from the oviduct after mating (Rankin et al., 1996), and early embryos whose zonae have been enzymatically removed are unable to traverse the oviduct (Bronson and McLaren, 1970; Modlinski, 1970).

These observations make clear that the zona pellucida plays critical roles in mammalian fertilization and early embryogenesis. However, the role of individual proteins in these processes has yet to be satisfactorily resolved and there is no consensus of experimental data as to whether a single zona protein, or a combination of zona proteins (or their post-translational modifications), control the specificity of sperm-egg interactions. Eutherian mammals are separated evolutionarily by less than 130 million years (Kumar and Hedges, 1998) and individual zona proteins are well conserved among them. Mammals have internal fertilization and speciation is adequately maintained by pre-mating (habitat, mate discrimination, physiognomy) and post-mating (hybrid lethality, sterility) determinants (O'Rand, 1988). Thus, despite the lack of concordance in the current literature, it seems unlikely that different classes of eutherian mammals would utilize different zona proteins for sperm binding.

In the mouse, each zona protein is unique and encoded by single copy genes located on different somatic chromosomes (Epifano et al., 1995b; Liang et al., 1990; Kinloch et al., 1988; Chamberlin and Dean, 1989; Lunsford et al., 1990). *Zp1* and *Zp2* appear to have arisen from a partial duplication of a common ancestral gene and all three proteins share a 260 amino acid 'zona domain' (Bork and Sander, 1992) near their carboxyl termini (Epifano et al., 1995a,b), postulated to play a role in extracellular matrix formation (Killick et al., 1995; Legan et al., 1997). A current structural model of the mouse zona suggests that dimers of ZP1 serve to cross-link heterodimeric filaments of ZP2 and ZP3 (Greve and Wassarman, 1985; Green, 1997). Other vertebrates, notably teleostean fish, form an egg shell composed of homologous proteins (Lyons et al., 1993; Murata et al., 1995; Chang et al., 1996, 1997; Murata et al., 1997; Del Giacco et al., 1998; Sugiyama et al., 1998) to which sperm do not bind but rather penetrate via a funnel shaped micropyle (Yamagami et al., 1992; Amanze and Iyengar, 1990). Although both ZP2 and ZP3 have been described as involved in mouse sperm binding in vitro (Bleil and Wassarman, 1980; Bleil et al., 1988), the function of ZP1 in mouse fertilization is less well defined. To further investigate the role of ZP1 in vivo, we have created mutant mice lacking ZP1 using targeted mutagenesis in embryonic stem cells.

## MATERIALS AND METHODS

### Gene targeting

A targeting construct was made using *Zp1* isolated from 129/Sv mouse genomic DNA (Epifano et al., 1995b). A 9.2 kb *Bam*HI-*Eco*RI fragment, extending from the middle of exon 3 into the 3' flanking region of *Zp1*, was ligated into the multi-cloning site of pPNT (Tybulewicz et al., 1991) located between the PGK-Neo and PGK-TK cassettes. A 3.0 kb fragment, extending from the *Bam*HI site approximately 2.9 kb 5' to the transcription start site to the *Xho*I site in exon 1 (+95 bp), was subcloned into Bluescript II KS (Stratagene). A second *Xho*I restriction site was added immediately 5' to the *Bam*HI site of this fragment with a synthetic oligonucleotide ligated into the *Not*I, *Bam*HI sites. To complete the targeting construct, the 3.0 kb *Xho*I fragment was excised from Bluescript and ligated into the *Xho*I site of pPNT 5' to the PGK-Neo cassette. After linearization at the 5' *Not*I site, the *Zp1* targeting construct was electroporated into R1 embryonic stem cells (Nagy et al., 1993) and individual clones were selected after growth in G418 (Gibco) and gancyclovir (Roche Discovery Welwyn). Four percent of the clones were correctly targeted based on Southern blot analysis with a 0.5 kb <sup>32</sup>P-labeled probe 5' to the end of the targeting construct that hybridized with a 7 kb normal and 5 kb mutant allele after digestion with *Eco*RV. Identity of the mutant allele was confirmed by hybridization with a <sup>32</sup>P-labeled 0.6 kb *Pst*I fragment isolated from PGK-Neo. Heterozygous *Zp1*<sup>tm/+</sup> cells from multiple targeted cell lines were injected into C57BL/6N blastocysts to obtain coat color chimeras and germ line transmission was assayed by Southern blot analysis of DNA isolated from tails (Rankin et al., 1996).

### In situ hybridization

Ovaries were isolated from 3-week-old *Zp1*<sup>tm/tm</sup> and normal females and fixed with 4% paraformaldehyde in 0.1 M sodium cacodylate, pH 7.2 for 5 hours to overnight at room temperature. Tissues were dehydrated, embedded in paraffin (American Histolabs) and sections (4-6 µm) were placed on silanated slides. <sup>35</sup>S-labeled sense and anti-sense RNA probes were generated from cDNA clones of ZP1, ZP2

and ZP3 and in situ hybridization was performed (Rankin et al., 1996). After 4 or 10 days of exposure, slides were developed with Dektol developer (diluted 1:1 with water) fixed with Kodak fixative and counterstained with hematoxylin.

### Western blot analysis

Ovulated eggs from 4- to 6-week-old *Zp1*<sup>tm/tm</sup> (20 eggs) and normal (10 eggs) lines were dissolved in sample buffer, separated by 4-20% SDS-PAGE (Laemmli, 1970) and transferred onto a nitrocellulose membrane (Burnette, 1981). Samples to be probed with antibodies to ZP3 were prepared with 5% β-mercaptoethanol; those to be probed with antibodies to ZP1 and ZP2 were not. After incubation of the blot with monoclonal antibodies (1:1000 for antibodies to ZP1 and ZP3, 1:20,000 for antibodies to ZP2, 1 hour, 20°C) specific to mouse ZP1 (M1.4), mouse ZP2 (IE-3) and mouse ZP3 (IE-10; Rankin et al., 1998; East and Dean, 1984; East et al., 1985), zona proteins were detected with a biotinylated goat anti-rat IgG (1:1000, Jackson Immunoresearch) followed by incubation in the Vectastain ABC reagent. Reactive proteins were visualized using a chemiluminescence detection system (Amersham) and XOMat AR X-ray film (Kodak). Biotinylated proteins (Bio-Rad) provided protein standards for molecular masses in the SDS-PAGE.

### Ovarian histology

Ovaries were isolated from *Zp1*<sup>tm/tm</sup> and normal 3-week-old females, fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 overnight, rinsed in the same and transferred to 70% ethanol. Tissues were dehydrated and embedded in methacrylate and 2 µm sections cut (American Histolabs). Mounted sections were stained with periodic-acid Schiff's reagent (PAS) and hematoxylin. Additional mice were stimulated by injection with 5 IU pregnant mares serum gonadotrophin (PMSG)/animal and 48 hours later with 5 IU of human chorionic gonadotrophin (hCG)/animal (Sigma). These mice were killed 9 hours later and their ovaries were prepared and sectioned as described to observe pre-ovulatory follicles.

### Isolation of eggs and embryos

*Zp1*<sup>tm/tm</sup> and normal females were stimulated with gonadotrophins as described above. To obtain ovulated eggs, mice were killed 14 hours after administration of hCG and isolated cumulus masses were dissociated by treatment with type IV-S hyaluronidase (300 µg/ml; Sigma) for 2-3 minutes. To obtain two-cell embryos, hormone-stimulated mice were mated with males proven to be fertile, and mice with visible vaginal plugs were killed at 40 hours. Embryos were collected by flushing the oviduct/uterus with M2 medium (Specialty Media).

### Electron microscopy

Ovaries were removed from gonadotrophin-stimulated females 10 hours after hCG injection and placed in Earle's balanced salt solution containing 0.1% polyvinylpyrrolidone (PVP) (EBSS/PVP). Mature follicles were ruptured with a pin and the oocyte-cumulus complexes were isolated and washed in EBSS/PVP. The complexes were fixed in glutaraldehyde and osmium tetroxide containing 0.5% ruthenium red. After embedding in Spurr's plastic, thin sections were examined with a Hitachi H-500 transmission electron microscope. Negatives were scanned with a Microtek scanner, and digital images were processed with Adobe Photoshop (Talbot and DiCarantonio, 1984).

For scanning electron microscopy, cumulus cells were released with hyaluronidase (see above) and the oocytes were washed in EBSS/PVP. The cumulus-free oocytes were fixed as described except that ruthenium red was omitted from the fixatives and during glutaraldehyde fixation, oocytes were attached to poly-L-lysine coated coverslips for subsequent processing. After dehydration, oocytes were dried with CO<sub>2</sub> in a Samdri PVT-3 critical point drier, mounted on aluminum pedestals, coated with gold/palladium in an Emscope coater (SC500), and examined on a Phillips XL-30 FEG scanning

electron microscope. Digital images were captured directly with the microscope, then processed and analyzed using Photoimpact (Ulead Systems) or Adobe Photoshop.

### Sperm-binding and in vitro fertilization

Epididymal mouse sperm were isolated from retired male breeders and capacitated by incubation with Eagle's Minimum Essential Medium (MEM) supplemented with 3% BSA for 1 hour at 37°C (Ho et al., 1995). To assay sperm binding, unfertilized eggs (from *Zp1<sup>tm/tm</sup>* and normal mice) were incubated with 500,000/ml motile, capacitated mouse sperm in modified MEM (20  $\mu$ l) 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 control two-cell mouse embryos possessed 2-3 sperm/embryo, then fixed for 2 hours in 1% paraformaldehyde/2% PVP in PBS, and mounted to quantify adherent sperm by differential interference contrast microscopy (Bleil and Wassarman, 1980). In each experiment, sperm binding to 10-14 null or normal eggs was determined and each experiment was performed in triplicate.

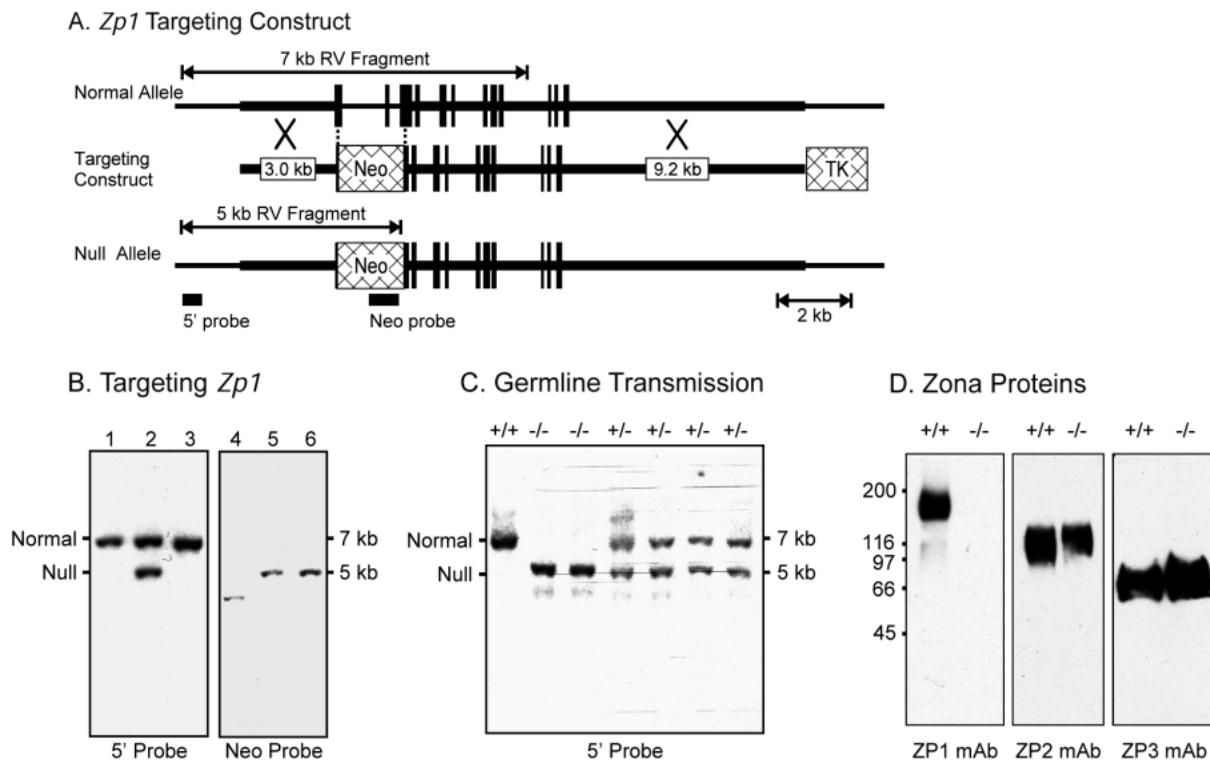
For in vitro fertilization, isolated cumulus masses were incubated in 0.5 ml MEM supplemented with 3% BSA (Ho et al., 1995) in the presence of capacitated sperm. At 6 hours post-insemination, the zygotes were washed into fresh M2 medium (Specialty Media) and scored for the presence of second polar bodies and male pronuclei.

## RESULTS

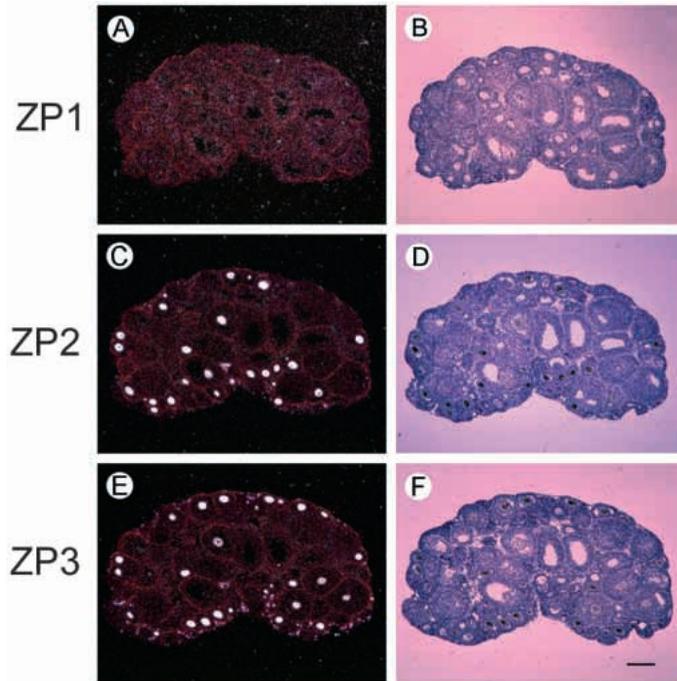
### Targeted mutagenesis of *Zp1*

Mouse *Zp1* was isolated from a 129/Sv mouse genomic library and used as source DNA to design a targeting vector for the endogenous single-copy gene located on Chromosome 19 (Epifano et al., 1995b). The construct (Fig. 1A), containing 12 kb of isogenic DNA (3.0 kb 5' to the transcription start site and 9.2 kb extending from exon 3 into the 3' flanking region), successfully targeted *Zp1* (Fig. 1B) in 4% of the R1 embryonic stem cells isolated after positive-negative selection (Thomas and Capecchi, 1987). Three independently targeted cell lines were injected into C57Bl/6 host blastocysts to derive mouse lines. Coat color chimerae were bred to CF-1 females to obtain F<sub>1</sub> heterozygotes. Both male and female F<sub>1</sub> heterozygotes were fertile and, when mated, produced F<sub>2</sub> normal (*Zp1<sup>+/+</sup>*), heterozygous (*Zp1<sup>tm/+</sup>*) and homozygous mutant (*Zp1<sup>tm/tm</sup>*) offspring in the expected Mendelian ratios for a mutation in a single-copy gene (Fig. 1C).

Creation of a *Zp1* null allele was confirmed by in situ hybridization using <sup>35</sup>S-labeled antisense probes and ovarian sections isolated from normal and mutant ovaries. No ZP1



**Fig. 1.** Targeted mutagenesis of the *Zp1* gene. (A) Top, schematic representation of the normal *Zp1* allele with twelve exons on chromosome 19. Middle, the targeting construct with PGK-Neo and PGK-TK as positive and negative selectable markers, respectively. Bottom, the *Zp1* allele mutated by homologous recombination. Vertical boxes represent exons; *EcoRV* fragments are indicated above the normal and null alleles; thicker lines indicate extent of homologous DNA. (B) Genotyping of embryonic stem cells by Southern blots of purified DNA hybridized with 5' (left) and neo (right) probes. After digestion with *EcoRV* the normal and mutant alleles detected with the 5' probe had restriction enzyme fragments of 7.0 and 5.0 kb, respectively. Lanes 2 and 5 targeted RI cells (clone TR31); lanes 1,3,4 and 6 mistargeted RI cells. (C) Genotyping by Southern blot analysis of DNA purified from tails of F<sub>2</sub> females generated from a *Zp1<sup>tm/+</sup>* × *Zp1<sup>tm/+</sup>* cross after restriction digest with *EcoRV* and hybridization with the 5' probe. Normal *Zp1<sup>+/+</sup>* (+/+), heterozygous *Zp1<sup>tm/+</sup>* (+/-) and homozygous *Zp1* null (-/-) were present in the expected Mendelian ratios of a single copy mutant gene. (D) Western blot of ovulated eggs isolated from *Zp1* null (-/-) and normal (+/+) mice after incubation with monoclonal antibodies specific to mouse ZP1, ZP2 or ZP3. 20 null or 10 normal eggs were used per lane. Molecular mass markers (kDa) are on the left.



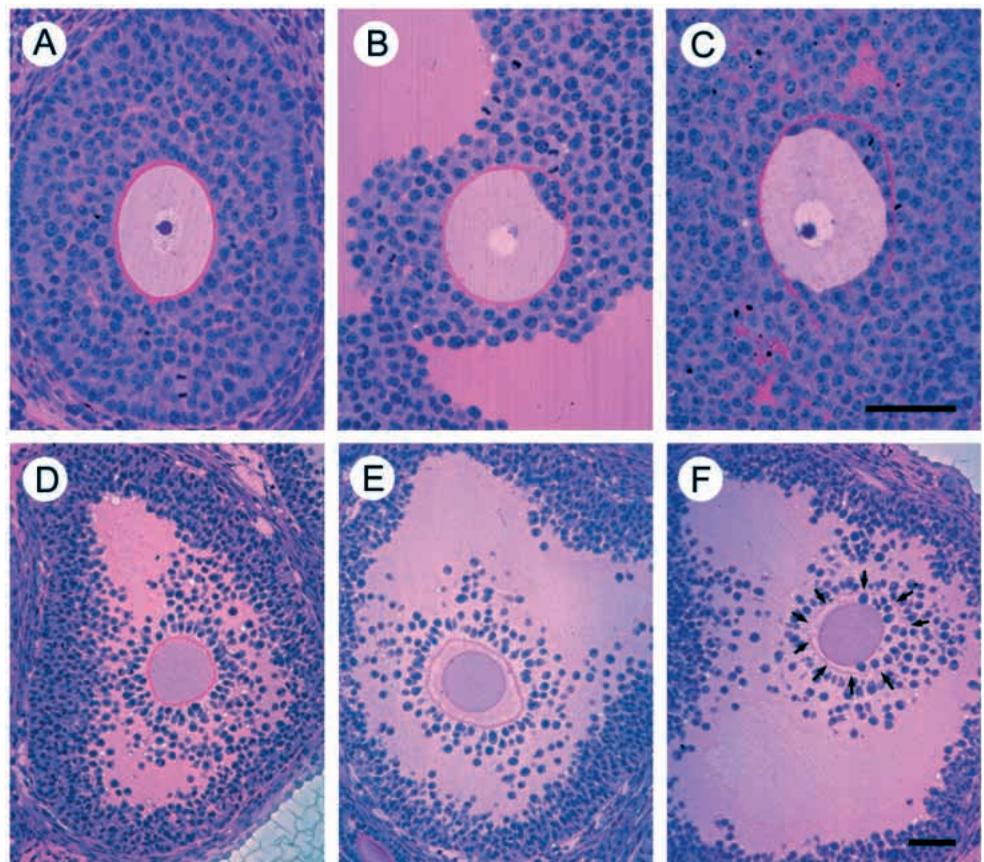
**Fig. 2.** In situ hybridization of *Zp1<sup>tm/tm</sup>* ovaries. <sup>35</sup>S-labeled anti-sense RNA probes derived from ZP1 (A,B), ZP2 (C,D) and ZP3 (E,F) cDNAs were hybridized to paraffin wax embedded ovarian sections isolated from 3-week-old mice and viewed under dark-field (A,C,E) and bright-field (B,D,F) optics. Probes specific to ZP2 (C,D) and ZP3 (E,F) but not ZP1 (A,B) hybridized to oocytes, within growing follicles throughout the ovary. Scale bar, 200  $\mu$ m.

transcripts were detected in *Zp1<sup>tm/tm</sup>* ovaries (Fig. 2A,B), although both ZP2 (Fig. 2C,D) and ZP3 (Fig. 2E,F) transcripts were present in all growing oocytes. All three zona transcripts were detected in normal ovaries and control sense probes gave backgrounds comparable to the negative data in Fig. 2A (data not shown). The absence of a *Zp1* gene product was confirmed by western blot analysis of ovulated eggs from normal and *Zp1<sup>tm/tm</sup>* animals in which ZP1 protein was detected in normal but not *Zp1<sup>tm/tm</sup>* eggs using a monoclonal antibody specific to mouse ZP1 (Fig. 1D). Two independently derived mouse lines with germline transmission, *Zp1<sup>tm1Nih/tm1Nih</sup>* and *Zp1<sup>tm2Nih/tm2Nih</sup>*, have been stably maintained for over a year.

#### Ovarian histology of *Zp1* null mice

Both male and female *Zp1* null mice appeared normal and the ovaries of *Zp1* null females were grossly indistinguishable from normal litter mates. When *Zp1* null ovaries were examined histologically, follicles at all stages were detected including corpora lutea, indicative of past ovulations. Significantly, all of the oocytes within the *Zp1* null ovary were surrounded by a zona pellucida, which contrasts with *Zp3* null females, in which growing oocytes fail to form a zona matrix in the absence of ZP3 protein (Liu et al., 1996; Rankin et al., 1996). The zona surrounding *Zp1* null mice contained ZP2 and ZP3 as determined by immunohistochemistry (data not shown), and western blot analysis (Fig. 1D) suggested that the ratio of the two proteins is no different than that observed in normal mice. The majority of follicles in *Zp1* null mice had normal architecture (Fig. 3A). However, in approximately 10% of the growing follicles, granulosa cells of varying numbers

**Fig. 3.** Ovarian histology of *Zp1* null females. Ovaries isolated from *Zp1<sup>tm/tm</sup>* mice, either in the absence of gonadotrophin stimulation (A-C) or 9 hours post-hCG (D-F), were fixed in 3% glutaraldehyde and stained with periodic-acid Schiff's reagent and hematoxylin. (A) Most *Zp1<sup>tm/tm</sup>* growing follicles had normal follicular architecture. In approximately 10% of the follicles, ectopic clusters of granulosa cells were juxtaposed between the inner aspect of the zona pellucida matrix and the plasma membrane either as single (B) or multiple (C) loci. (D) Most *Zp1<sup>tm/tm</sup>* pre-ovulatory follicles had post-meiotic eggs that were surrounded by a zona that was sufficient to organize a normal appearing oocyte-cumulus complex. Some pre-ovulatory follicles had abnormally large perivitelline spaces that contained PAS-staining material (E) and others (F) in which the perivitelline space was occupied by a large number of granulosa cells had a grossly disorganized zona pellucida (arrows). Scale bars, 50  $\mu$ m.



were detected between the zona matrix and the oolemma (Fig. 3B,C). This phenomenon was most apparent in those follicles at the late secondary stage and could be quite dramatic, resulting in increased disorganization and consequent dispersal of the zona matrix.

The structural abnormality of the zona pellucida was even more striking in pre-ovulatory follicles. Normally, the oocyte completes the first meiotic division just prior to ovulation and, together with the innermost layers of granulosa cells (cumulus cells), forms the oocyte-cumulus complex. During the mucification reaction, the cumulus cells secrete a viscous extracellular matrix that dissociates them one from another, but they remain a structural unit with the egg because of cellular processes that tether them to the zona matrix (Eppig, 1991). In all *Zp1<sup>tm/m</sup>* follicles, oocytes reached full size and completed the first meiotic division. Many follicles underwent the mucification reaction to form well-organized oocyte-cumulus complexes in which the rest of the follicular architecture appeared normal (Fig. 3D). However, in some follicles, the perivitelline space between the egg and zona was grossly accentuated and filled with lightly PAS-staining material (Fig. 3E). The cumulus cells continued to be well organized around these oocytes, but the zona appeared to be stretched during the mucification reaction. In follicles in which cumulus cells were present in the perivitelline space, the zona matrix was further distorted and only barely evident (Fig. 3F, arrows). These extreme phenotypes may reflect concomitant stretching by cumulus cells attached on the outside of the zona and pressure from the extracellular matrix being secreted by the cumulus cells on the inside. Together, these data suggest that not only is the structural integrity of the zona compromised by the absence of ZP1, but that the *Zp1* null matrix is less well associated with oocytes after gonadotrophin-induced maturation.

### Structure of zonae pellucidae lacking ZP1

To further define the structural abnormality of the zona pellucida in *Zp1* null mice, oocytes were isolated from mature follicles and viewed by scanning electron microscopy (Fig. 4). In all oocytes examined, the zona matrix was present, but somewhat thinner than normal. The *Zp1* null matrix had larger fenestrations (compare Fig. 4A,C with D,F), and one could observe the egg plasma membrane through these pores (Fig. 4C, arrow). Additionally, the zona matrix of the *Zp1* null

females appeared to adhere to granulosa cells and extend in between them, a phenomenon not observed in normal zonae (compare Fig. 4B and E). Such engulfment during zona formation could result in ectopic localization of granulosa cells within the perivitelline space.

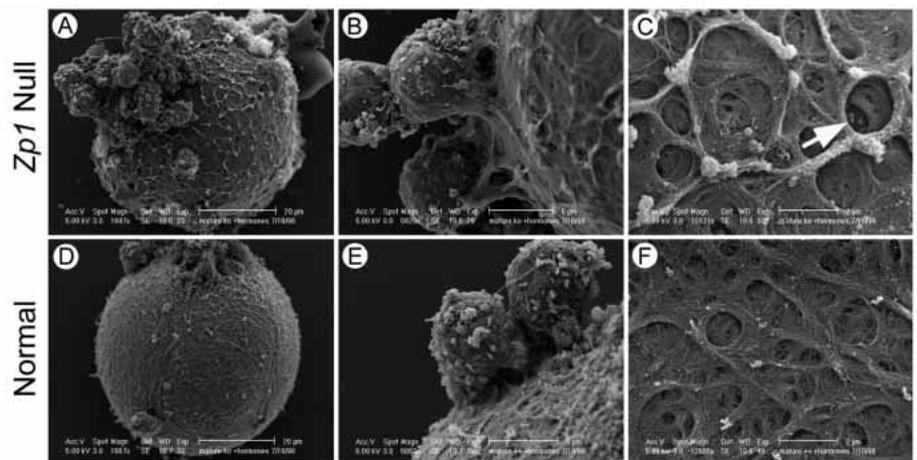
Ovulated eggs from *Zp1* null and normal females were isolated after gonadotrophin stimulation. By western blot analysis there was no detectable ZP1 in the null eggs (Fig. 1D) and thus, it appears that ZP2 and ZP3 were sufficient to form a zona matrix around the *Zp1* null eggs. After fixation with 1% paraformaldehyde, the null and normal eggs were examined by light microscopy using Nomarski optics. Although most *Zp1* null ovulated eggs were encased in zonae pellucidae, their zona matrices were often thinner with a poorly defined peripheral border compared to normal eggs (Fig. 5A). These abnormalities were further defined ultrastructurally by transmission electron microscopy of expanded oocyte-cumulus complexes in the presence of ruthenium red to accentuate the zona matrix.

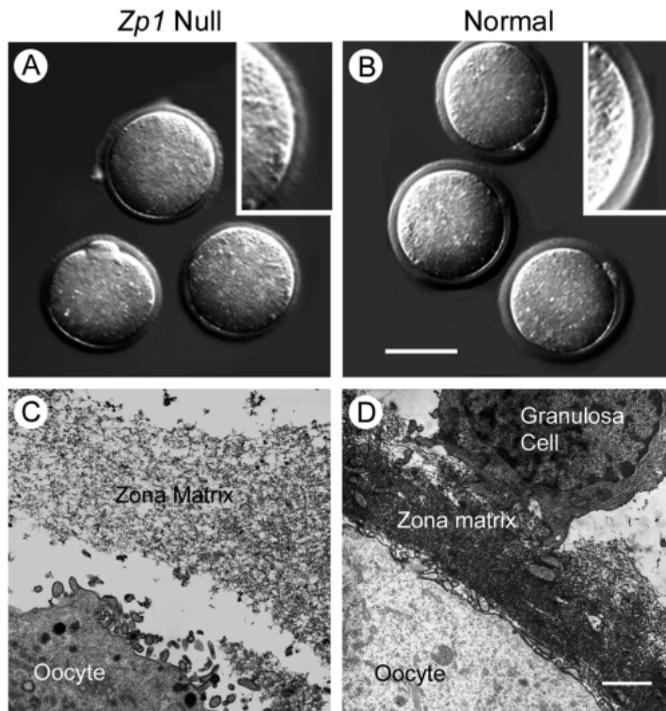
In normal oocytes, the zona matrix was composed of stout filaments and small, electron dense granules most readily observed at the outer periphery where the matrix was less compact. Here the filaments were well separated from each other and appeared to link together to form 4-6 sided structures with granules often seen where the filaments joined one another. Moving toward the surface of the oocyte, the zona pellucida became increasingly dense which obscured structural detail (Fig. 5D). The *Zp1* null zonae were also composed of stout filaments and small electron dense granules, but these structures were quite dispersed and not compacted together in any region of the matrix, including that nearest the oocyte (Fig. 5C). As was observed by light microscopy, the perivitelline space in the *Zp1* null oocytes was enlarged compared to normal and, in some oocyte-cumulus complexes, cumulus cells were observed between the zona matrix and the oolemma. These 'trapped' cells had undergone mucification and a granular matrix was observed surrounding them and infiltrating the zona matrix (data not shown). As noted above, these cumulus cell secretions may contribute to distortion of the zona matrix observed in pre-ovulatory follicles.

### Fertility of *Zp1* null mice

*Zp1* null and normal female mice were stimulated with gonadotrophins to obtain ovulated eggs from their oviducts. All

**Fig. 4.** Scanning electron microscopy of *Zp1* null and normal mice. Cumulus-free oocytes were prepared after isolation from follicles of gonadotrophin stimulated *Zp1* null (A-C) and normal (D-F) mice. The zona matrix from *Zp1* null mice (A,C) possessed large fenestrations compared to normal (D,F) and the oolemma could be observed through these enlarged pores (C, arrow). Additionally, the *Zp1* null matrix was found in between the cumulus cells (B), a phenomenon not observed with normal zonae (E) and could result in ectopic location of granulosa cells within the perivitelline space.





**Fig. 5.** Structure of the *Zp1* null and normal zonae pellucidae. Ovulated eggs were collected from *Zp1* null (A) or normal (B) females, fixed in 1% paraformaldehyde and visualized under Nomarski optics. The *Zp1* null zonae pellucidae appeared thinner with poorly defined peripheral borders compared to normal eggs. Magnification of insets (A and B) was 2 $\times$ . (C,D) Oocyte-cumulus cell complexes isolated from follicles of gonadotrophin-stimulated mice were processed for transmission electron microscopy. The zona matrix in *Zp1* null animals (C) was composed of stout filaments with a loose weave that were not compacted together as they were in the more electron dense matrix seen in normal females (D). Additionally, the perivitelline space surrounding *Zp1* null oocytes was considerably larger than in normal mice and the zona matrix had, for the most part, moved off the surface of the oocyte. Scale bars, 50  $\mu$ m (A,B); 1  $\mu$ m (C,D).

null and normal mice responded to hormone stimulation. Although fewer eggs were isolated from *Zp1* null (18.5 $\pm$ 3.0) compared to normal (23.6 $\pm$ 3.9) mice, there was no statistical difference between the two groups (Table 1). However, the zona surrounding many of the *Zp1* null eggs was not normal. The predominant abnormality in *Zp1* null eggs was the accentuated perivitelline space observed during folliculogenesis, but there were also a number of zona ghosts indicative of egg lysis or escape (Fig. 6A). Despite abnormal zona matrices, 80% of the *Zp1* null females were fertile. However, these fertile animals consistently gave birth to litters that were about half the size of those from normal females (Table 1). Since equivalent numbers of ovulated eggs were retrieved from *Zp1* null and normal mice, the cause of the decreased fecundity most likely reflected sub-optimal fertilization or early embryonic loss. The latter could result from polyspermy or precocious hatching from the structurally abnormal zona pellucida surrounding the *Zp1* null eggs.

To assay for sperm-binding and in vitro fertilization, ovulated eggs were collected from *Zp1* null and normal mice. Although in vitro fertilization was equally successful using

**Table 1.** Number of ovulated eggs, two-cell embryos and live births

	Ovulated eggs	2-Cell embryos	Live births
<i>Zp1</i> Null ( <i>Zp1<sup>tm/tm</sup></i> )	18.5 $\pm$ 3.0 (12)*	5.7 $\pm$ 2.4 (12)	3.6 $\pm$ 0.4 (20)‡
Normal ( <i>Zp1<sup>+/+</sup></i> )	23.6 $\pm$ 3.9 (13)	22.4 $\pm$ 6.2 (10)	7.3 $\pm$ 0.7 (23)

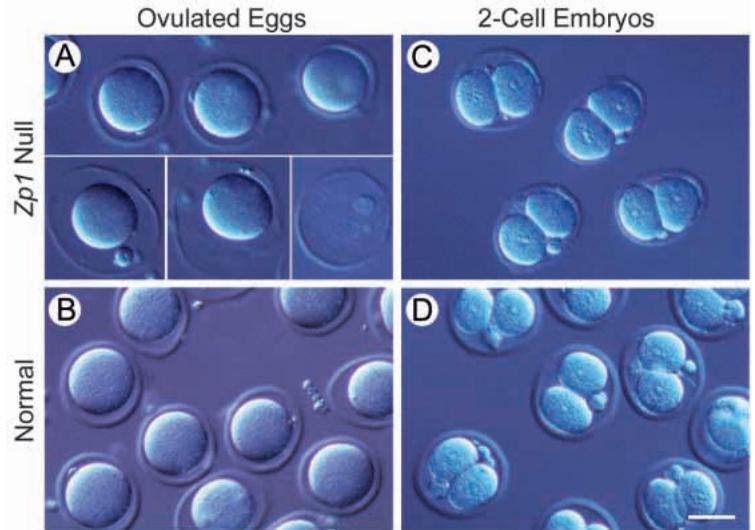
\*avg.  $\pm$  s.e.m. (number of animals).  
‡avg.  $\pm$  s.e.m. (number of litters).

eggs ovulated by *Zp1* null and normal mice, there was a statistically significant ( $P<0.05$ ) increase in the average number of sperm bound to null (37.9 $\pm$ 4.4) compared to normal (18.9 $\pm$ 2.2) zonae pellucidae. Sperm appeared to bind equally well to different *Zp1* zonae whether or not the perivitelline space was enlarged and no sperm were observed within the perivitelline space. The basis for the increased sperm binding was unclear, but may reflect additional sperm binding sites available in the looser weave reflected in decreased electron density of the zona matrix of *Zp1* null mice. After in vivo fertilization, one-cell zygotes were examined microscopically for extra pronuclei and no increase in polyspermy was observed in *Zp1* null embryos compared to normal.

It has been previously reported that biochemical (Bronson and McLaren, 1970; Modlinski, 1970) or genetic (Liu et al., 1996; Rankin et al., 1996) removal of the zona pellucida prevents passage of the early embryo (<8 cells) through the oviduct. To determine if the observed fragility in the *Zp1* null zonae could account for decreased litter sizes, *Zp1* null and normal mice were stimulated with gonadotrophins and mated in vivo. Forty hours after administration of hCG, their oviducts were flushed to recover early embryos. The number of 2-cell embryos recovered from the *Zp1* null mice was dramatically decreased compared the number of embryos recovered from normal mice (5.7 $\pm$ 2.4 versus 22.4 $\pm$ 6.2; Table 1), but none of the zonae was distorted like those surrounding ovulated eggs (Fig. 6A). These observations are consistent with *Zp1* eggs encased in distorted zonae being unable to progress to the 2-cell embryo stage. Thus, only those *Zp1<sup>tm/tm</sup>* embryos with a structurally competent zona that made it to the 2-cell stage would pass down the oviduct and implant in the uterus. This early embryonic loss could account for the observed decrease in litter size (Table 1).

## DISCUSSION

The zonae pellucidae of all eutherian mammals investigated are composed of three major glycoproteins that correspond to mouse ZP1, ZP2 and ZP3. Mice lacking ZP3, but expressing ZP1 and ZP2, do not form a zona pellucida and are infertile (Liu et al., 1996; Rankin et al., 1996). We have now created genetically altered mice lacking ZP1. Although they form a zona pellucida composed of ZP2 and ZP3 and although most (80%) females are fertile, they have litters of half the normal size. After fixation, the mutant zonae appear thinner than normal and their structural integrity is compromised due to a looser weave in the matrix. This results in increased fragility and distortion of the zona pellucida which can lead to ectopic accumulation of granulosa cells within the perivitelline space. However, the striking decrease in fecundity in the ZP1



**Fig. 6.** Decreased fecundity of *Zp1* null mice. Unfixed ovulated eggs from *Zp1* null and normal mice were examined by Nomarski optics. Although similar numbers of eggs were recovered from the oviduct, the zonae pellucidae lacking ZP1 (A) were quite heterogeneous compared to normal (B). Although some *Zp1* null zonae appeared quite normal (A, upper), others were distorted with dramatically enlarged perivitelline spaces and a few appeared as wraiths lacking eggs (A, lower). After *in vivo* mating, two cell embryos were flushed from *Zp1* null (C) and normal (D) mice. Half as many embryos were recovered from the *Zp1* null mice compared to normal (Table 1), but none was surrounded by grossly distorted zonae pellucidae. Scale bar, 50  $\mu$ m.

null females cannot be attributed solely to abnormal folliculogenesis as equivalent numbers of eggs are ovulated by normal and ZP1 null animals. Furthermore, the unimpaired ability of sperm to bind to the ZP2/ZP3 zona pellucida and fertilize eggs (*in vitro* and *in vivo*), indicates that ZP1 is not absolutely required for sperm binding. Rather, the decrease in fecundity appears to reflect precocious hatching of eggs and early embryos from an abnormal zona matrix. These data suggest that ZP1 acts primarily to augment the structural integrity of the zona matrix, without which the early embryo cannot survive in the oviduct at the very earliest stages of embryogenesis.

An understanding of the molecular basis of sperm binding to the zona pellucida remains elusive, even though key components have been identified. All vertebrates studied to date have an egg shell that mediates initial sperm-egg interactions and contains proteins homologous to the three mouse zona proteins. Each of the three classes of protein, including ZP1, has been suggested in at least one vertebrate to mediate sperm binding *in vitro*, but there seems to be little consensus among different species. In mouse, both ZP2 and ZP3 have been implicated in the initial steps of gamete recognition. The most compelling evidence is that ZP3 (but not ZP1 or ZP2) purified by SDS-PAGE inhibits *in vitro* sperm binding to eggs in a dose-dependent manner (Bleil and Wassarman, 1980). The initial binding of sperm to the zona triggers the acrosome reaction that releases lytic enzymes thought to modify the sperm or the zona matrix to facilitate continued binding and penetration of the zona pellucida. Paradoxically, sperm that have spontaneously undergone the acrosome reaction, do not bind to the zona pellucida (Saling et al., 1979; Florman and Storey, 1982).

The evidence that ZP2 acts as a sperm binding macromolecule in mice comes from morphometric studies in mice and the observed ability of antibodies to prevent the maintenance of sperm binding (Bleil and Wassarman, 1986; Bleil et al., 1988). More persuasive is the observation in *Xenopus laevis* that the SDS-PAGE purified homologue of ZP2 (gp69/64), but not the homologue of ZP3 (gp43/41) or ZP1 (gp37), effectively inhibits *Xenopus* sperm binding to *Xenopus* eggs *in vitro* (Tian et al., 1997a). Following fertilization, sperm

no longer bind to the egg shell of mammals or frogs, and this post-fertilization block to polyspermy is temporally correlated with proteolytic cleavage of the N terminus of ZP2 and gp69/64 (Bleil et al., 1981; Tian et al., 1997b). An attractive and simple hypothesis is that sperm bind to intact ZP2 (or gp69/64) which is modified after fertilization to prevent additional sperm binding (Tian et al., 1999). However, concomitant subtle modifications of other zona proteins may also be important, or the proteolytic cleavage could induce global rearrangements of the zona matrix that preclude sperm binding.

There are also data that implicate ZP1 in the binding of rabbit and pig sperm to their cognate zonae, either by itself or in conjunction with ZP3. Recombinant rabbit ZP1 (r55) binds to the anterior surface of the sperm acrosome and antibodies to the protein inhibit sperm binding (Prasad et al., 1996). When expressed as recombinant protein, rabbit sp17, a sperm protein implicated in zona binding, can bind to either rabbit ZP1 (r55) or ZP3 (r45) raising the possibility of more than one binding molecule within the zona matrix (Yamasaki et al., 1995). Earlier studies implicated the pig ZP1 homologue (variously designated ZPB or ZP3 $\alpha$ ) in sperm binding (Sacco et al., 1989; Yonezawa et al., 1995) but most recently, it has been reported that neither pig ZP1 (ZPB) or ZP3 (ZPC) have sperm binding activity by themselves, but can form heteroduplexes *in vitro* that bind avidly to pig sperm membranes (Yurewicz et al., 1998).

Our current results with *Zp1* null mice indicate that a zona matrix can be formed with only ZP2 and ZP3 and that ZP1 is not required for fertilization in mice. These results suggest that mouse ZP2 and ZP3 are sufficient for sperm binding *in vitro* and fertilization *in vivo*, although these results do not preclude a facilitating function for ZP1, as postulated in other mammals (Yurewicz et al., 1998). We have also reported that human sperm will not bind to mouse eggs, even those obtained from transgenic mice in which human ZP3 replaces endogenous mouse ZP3 (i.e., zonae are composed of moZP1, moZP2 and huZP3). More surprisingly, despite the absence of mouse ZP3, these transgenic mice have normal fertility *in vivo* and mouse sperm bind and fertilize their eggs *in vitro*. Based on mobility in SDS-PAGE, human ZP3 expressed in the transgenic mouse

is post-translationally modified as human ZP3 (64 kDa) and distinct from mouse ZP3 (83 kDa) (Rankin et al., 1998). These results suggest that either ZP3 by itself is not critically important for the specificity of mouse and human sperm binding or that a subtle, as yet undefined, post-translational modification in mouse eggs functionally converts human ZP3 into mouse ZP3. Similar transgenic studies in which human ZP2 replaces endogenous mouse ZP2 should provide insight into the role of ZP2 in sperm-egg interactions.

All three zona proteins have a signal peptide to direct them into a secretory pathway and all contain transmembrane domains near their C termini that are preceded by a potential endo-proteolytic cleavage sites (Yurewicz et al., 1993; Epifano et al., 1995a). However, it remains unclear where the initial molecular association of the three zona proteins occurs in the secretory process and the mechanisms by which they form the insoluble, extracellular zona pellucida. As oocytes enter their growth phase, the zona matrix first appears as an amorphous material deposited in the space between the germ cell and the surrounding granulosa cells. This material is subsequently assembled into long filaments forming a highly porous matrix which increases in thickness to 7 µm as the diameter of oocytes grows from 12 µm to 80 µm (Phillips and Shalgi, 1980; Dietl, 1989). The enhanced fragility of the zona pellucida in the absence of ZP1 is consistent with a model of zona structure in which the matrix is composed of filaments of heterodimeric repeats of ZP2 and ZP3 cross-linked by dimers of ZP1 (Greve and Wassarman, 1985; Green, 1997). However, the ability of ZP2 and ZP3 to form a matrix around *Zp1* null eggs indicates that the biosynthesis of ZP2 and ZP3 and their ability to associate with each other is independent of ZP1. Individually, recombinant ZP2 or ZP3 are secreted from heterologous cells as soluble products that do not appear to self-aggregate (Beebe et al., 1992; Kinloch et al., 1991; Ann Ginsberg, personal communication). Theoretically, heterologous cells that co-express ZP2 and ZP3 should, like the *Zp1* null oocytes, be able to form an insoluble zona matrix, unless the absence of cell division, specific processing, high local concentrations of protein, or an additional protein are of critical import.

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