Development 122, 2903-2910 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
DEV3497

Mice homozygous for an insertional mutation in the *Zp3* gene lack a zona pellucida and are infertile

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SUMMARY

Mammalian oocytes synthesize and secrete a zona pellucida that surrounds the growing oocytes, ovulated eggs and preimplantation embryos. The extracellular zona matrix is composed of three glycoproteins (ZP1, ZP2, ZP3) that are involved in folliculogenesis, species-specific fertilization, and passage of the early embryo down the oviduct. We have established a mouse line in which *Zp3* has been inactivated by homologous recombination with an insertional mutation. Neither *Zp3* transcripts nor ZP3 protein was detected in female mice homozygous for the mutation (*Zp3*$^{-/-}$), whereas both ZP1 and ZP2 were present in mutant oocytes. Homozygous mutant *Zp3*$^{-/-}$ mice had follicles with germinal-vesicle-intact oocytes but that lacked a zona pellucida matrix and had a disorganized corona radiata. Although mutant oocytes underwent germinal vesicle breakdown (GVBD) prior to ovulation, the cumulus-oocyte complex was markedly disrupted and the oocytes were often separate from the cumulus cells. After hormone-induced ovulation, cumulus masses were present in the oviducts of homozygous mutant mice, but zona-free eggs were observed in only half of the females and, in these, less than 10% of the normal number of eggs were detected. No zona-free 2-cell embryos were recovered from homozygous mutant *Zp3*$^{-/-}$ female mice after mating with males proven to be fertile, and none became visibly pregnant or produced offspring. These results demonstrate that a genetic defect in a zona pellucida gene causes infertility and, given the conserved nature of the zona pellucida, a similar phenotype is expected in other mammals.

Key words: zona pellucida, infertility, ZP3 sperm receptor, zona-free oocytes, disruption of the corona radiata, *Zp3* null mutant mice

INTRODUCTION

The mammalian zona pellucida is synthesized by oocytes during folliculogenesis, an on-going cyclic process that begins just after birth and continues throughout the reproductive life of females. In mice, resting oocytes (12-15 μm), with no visible zona pellucida, are enclosed in a squamous epithelium of granulosa cells forming primordial follicles. As cohorts of follicles begin development, the granulosa cells become cuboidal and the zona pellucida becomes evident as extracellular patches that coalesce into a uniform matrix surrounding the growing oocyte. After approximately 12 days of growth, the 70-80 μm oocyte is encased in a 7 μm thick zona pellucida and surrounded by multiple layers of granulosa cells, together forming a 125 μm diameter follicle. The layer of granulosa cells closest to the oocyte constitutes the corona radiata. Although the growth of the oocyte slows dramatically over the next 3 days, the granulosa cells continue to proliferate, resulting in a 600 μm antral follicle containing a fully grown (75-85 μm) oocyte (Brambell, 1928).

The ultimate goal of folliculogenesis is to ovulate a mature egg for fertilization. Prior to ovulation, the cumulus-cell-enclosed oocyte projects into the antrum while remaining attached to the inner wall of the follicle by a cellular stalk. As a consequence of the luteinizing hormone (LH) peak in midcycle, the follicle becomes highly vascularized and the antrum is filled with a fluid rich in serum proteins. Oocytes undergo meiotic maturation and complete the first meiotic division, reaching metaphase II. In response to signals from the oocyte, the cumulus cells secrete a viscous extracellular matrix and dissociate from one another. However, the cumulus oophorus and oocyte remain a structural unit, the cumulus-oocyte complex, in which the granulosa cells radiate out from the centralized oocyte (Eppig, 1991). At ovulation, this complex is extruded into the oviduct and the egg is available for fertilization.

Although tens of millions of sperm are deposited at coitus, fewer than 20 approach the ovulated egg (Braden and Austin, 1954). One sperm must pass through the cumulus oophorus, penetrate the zona pellucida and fuse with the egg for successful fertilization. The binding of sperm to the zona pellucida induces the acrosome reaction that is required for zona penetration (Saling et al., 1979; Florman and Storey, 1982) and subsequent fusion of a sperm with the egg's plasma membrane (Yanagamachi, 1994). After fertilization, the zona is biochemically modified to prevent polyspermy (Sato, 1979; Bleil and Wassarman, 1980a; Bleil et al., 1981). Transfer studies in

which the zona pellucida was removed biochemically indicate that early embryos (fewer than 8 cells) require a zona pellucida to traverse the oviduct (Bronson and McLaren, 1970; Modlinski, 1970). Because of these essential roles, abnormalities in the zona matrix should be manifest during folliculogenesis, fertilization or early development. However, no genetic mutations resulting in an abnormal zona pellucida phenotype have been reported.

The mouse zona pellucida is composed of three sulfated glycoproteins (Bleil and Wassarman, 1980b; Shimizu et al., 1983) encoded by single copy genes (*Zp1*, *Zp2*, *Zp3*) located on Chromosomes 19, 7 and 5, respectively (Epifano et al., 1995a; Liang et al., 1990; Lunsford et al., 1990; Kinloch et al., 1988; Chamberlin and Dean, 1989). During oogenesis, ZP1, ZP2 and ZP3 mRNAs accumulate with a stoichiometry of 1:4:4 and in 50 μm diameter oocytes they represent 1.5% of the total poly(A)$^+$ RNA (Epifano et al., 1995b). The biosynthesis of the zona proteins appears to parallel the abundance of the zona transcripts and the incorporation of the zona proteins into the extracellular matrix occurs only during oogenesis (Bleil and Wassarman, 1980c; Shimizu et al., 1983). We have undertaken to disrupt the formation of the zona pellucida by introducing an insertional mutation into *Zp3* in embryonic stem cells (ES). These cells have been used to establish a *Zp3*$^{-/-}$ mutant mouse line in which females lack a zona pellucida.

MATERIALS AND METHODS

Gene targeting

A genomic clone containing the *Zp3* locus was obtained by screening a 129/Sv mouse genomic library (Stratagene) with mouse ZP3 cDNA (Ringuette et al., 1988). A 5.2 kb *Bam*HI fragment, including a portion of the promoter and exons 1-4 (–466 to +4697 bp) was isolated. *Bst*XI linkers were ligated to a 1.9 PGK-Neo cassette (McBurney et al., 1991) that was cloned into a *Bst*XI site in exon 1 (+134). A TK cassette (Mansour et al., 1988) was directionally cloned into *Xho*I and *Sac*I sites in a polylinker at the 3′ end of the genomic sequence. After linearization at a 5′ *Not*I site, the targeting construct was electroporated into J1 embryonic stem cells (Li et al., 1993). Individual clones were selected after growth in G418 (Gibco) and gancyclovir (Hoffman La Roche). 5% of the clones were correctly targeted based on Southern blot analysis with ^{32}P-labeled probes 5′ (–1518 to –943) and 3′ (cDNA encoding exons 5-8) to the targeting vector. Identity of the mutant allele was confirmed by hybridization with a ^{32}P-labeled 0.6 kb *Pst*I fragment isolated from PGK-Neo. Heterozygous *Zp3*$^{-/+}$ cells from one cell line (TR18) were injected into C57BL/6J blastocysts to obtain coat color chimeras and germ line transmission was assayed by Southern blot analysis of DNA isolated from tails (Accili et al., 1996).

Ovarian histology

Ovaries were isolated from 3-week old females, fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 for 3-5 hours, rinsed in the same and transferred to 70% ethanol. Tissues were dehydrated and embedded in methacrylate, and 2 μm sections were cut (American Histolabs). Mounted sections were stained with periodic-acid Schiff's reagent and hematoxylin.

In situ hybridization

Ovaries were isolated from 3-week old females and fixed in either Perfix solution (4% paraformaldedyde, 20% isopropanol, 2% zinc chloride and addition of trichloroacetic acid to pH 6) or in 2% paraformaldehyde in 0.1 M sodium cacodylate, pH 7.2 for 5 hours at room temperature. Tissues were transferred to 70% ethanol, dehydrated, embedded in paraffin (American Histolabs) and sections (4-6 μm) were placed on silanated slides. ^{35}S-labeled sense and anti-sense RNA probes were generated from cDNA clones of ZP1, ZP2 and ZP3 and in situ hybridization was performed as described (Epifano et al., 1995b) except hybridizations were carried out at 55°C overnight. After 4 or 10 days of exposure, slides were developed with Dektol developer (diluted 1:1 with water) and Kodak fixer, and counterstained with hematoxylin.

Immunocytochemistry with confocal microscopy

Oocytes were isolated by follicle puncture in Ca^{2+}-, Mg^{2+}-free Brinster's medium (NIH Medium Unit) supplemented with 0.3 mM sodium pyruvate, 25 mM Hepes buffer, pH 7.3 and 0.25 mM dibutyrl cAMP (Sigma), and fixed in 3% paraformaldehyde in PBS, pH 7.4 for 12 hours at room temperature. The fixed oocytes were rinsed three times in PBS, pH 7.4, quenched with glycine for 45 minutes and incubated for 1 hour with primary antibody (1:10) specific to ZP1 (Epifano et al., 1995b), ZP2 (East and Dean, 1984) or ZP3 (East et al., 1985). Cells were washed three times and incubated in rhodamine-lissamine conjugated goat anti-rat IgG (Jackson ImmunoResearch Labs) for 1 hour. All incubations were in PBS, pH 7.4, containing 1% saponin and 2.5 mg/ml goat IgG at room temperature. Following immunolabeling, oocytes were mounted in p-phenylenediamine glycerol (Johnson and Nogueira Araujo, 1981). Imaging was performed with a Nikon Optiphot microscope equipped with a BioRad MRC 1000 confocal imaging system and a krypton-argon laser. For detecting rhodamine, a 568 DF10 excitation filter and a 585 LP emission filter were used. Phase photos were taken on the same system using a single channel transmission detector.

Hormone stimulation of female mice

Zp3$^{+/+}$ and Zp3$^{-/-}$ females were injected with 5 IU PMSG/animal and 48 hours later with 5 IU of hCG/animal (Sigma). Groups of mice were killed after 9, 20 or 40 hours to observe pre-ovulatory follicles, ovulated eggs or 2-cell embryos, respectively. The ovaries were isolated and fixed in 3% glutaraldehyde, embedded and stained as described above. The cumulus mass was isolated from oviducts and viewed by light microscopy before and after treatment with type IV-S hyaluronidase (300 μg/ml; Sigma). The 2-cell embryos were flushed, using Brinster's medium, from oviducts of female mice with vaginal plugs after mating with fertile males.

RESULTS

Targeting the *Zp3* gene

A *Zp3* targeting vector was constructed from a 5.2 kb *Bam*HI fragment isolated from 129/Sv genomic DNA that contained the first 4 exons (–466 to +4697 bp) of *Zp3*. The 1.9 kb PGK-Neo cassette (McBurney et al., 1991) was inserted into the *Bst*XI site of exon 1 to disrupt the reading frame and provide a positive selectable marker. The 1.8 kb MC1-TK cassette (Mansour et al., 1988) was placed 3′ to the *Bam*HI fragment as a negative selectable marker (Fig. 1). After linearization, the targeting vector was electroporated into J1 ES cells (Li et al., 1993).

Following selection with G418 and gancyclovir, DNA from individual clones was analyzed for restriction fragment length polymorphisms on Southern blots with probes 5′ and 3′ of the targeting vector sequences (Fig. 1). ES cells from TR18, a cell line in which the targeting construct had integrated by homologous recombination, were microinjected into C57BL/6J blastocysts to obtain coat-color chimeras. In subsequent breeding

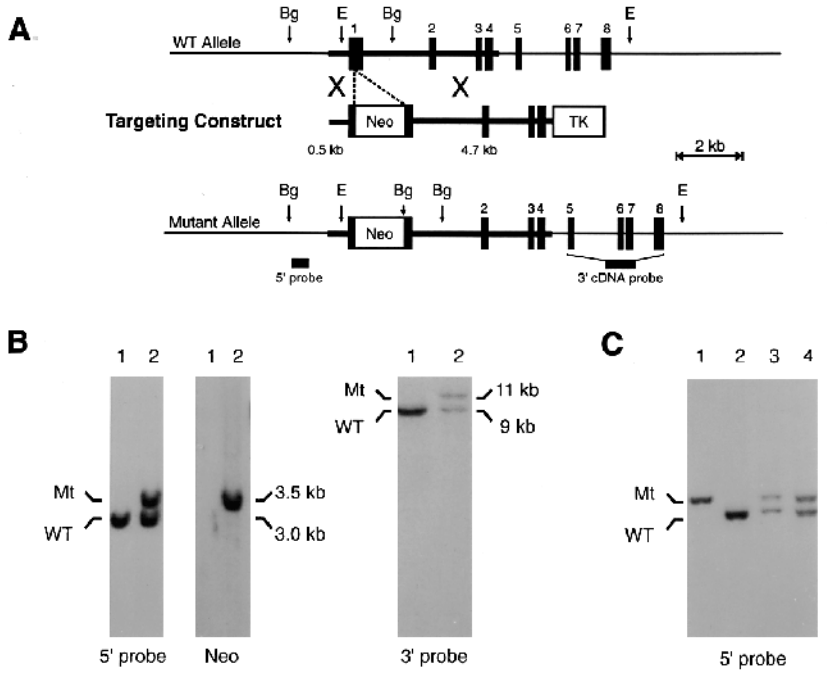

Fig. 1. Targeting the single copy *Zp3* locus on chromosome 5. (A) Schematic representation of normal *Zp3* allele (top), the targeting construct (middle), and the allele mutated by homologous recombination (bottom). Bg, *Bgl*II; E, *Eco*RI; numbers represent exons; thicker line indicates extent of homologous DNA. (B) Genotyping of embryonic stem cells by Southern blots of purified DNA probed with the 5′ (left), neo (middle) or 3′ probe (right). After digestion with *Bgl*II (left, center), the normal and mutant alleles detected with the 5′ probe had restriction enzyme fragments of 3.0 and 3.5 kb, respectively. After digestion with *Eco*RI (right), the normal and mutant DNA fragments detected with the 3′ probe were 9 and 11 kb, respectively. Lane 1, parental J1 cells; lane 2, targeted J1 cells (clone TR18). (C) Genotyping by Southern blot analysis of DNA purified from tails of F_2 females generated from a $Zp3^{-/+}$ x $Zp3^{-/+}$ cross after hybridization with the 5′ probe. 1, $Zp3^{-/-}$; 2, $Zp3^{+/+}$; 3,4 $Zp3^{+/-}$. Mutant allele (Mt); normal allele (WT).

with C57BL/6J, two coat-color chimeric males transmitted the *Zp3* mutation to their offspring. Both male and female F_1 heterozygotes were fertile, and when mated, produced F_2 normal ($Zp3^{+/+}$), heterozygous ($Zp3^{+/-}$) and homozygous mutant ($Zp3^{-/-}$) offspring in ratios expected from Mendelian genetics of a single copy gene (1:2:1; Fig. 1).

Ovarian histopathology

Both male and female $Zp3^{-/-}$ mice appeared normal and, at a gross anatomical level, the ovaries of the homozygous mutant mice were indistinguishable from $Zp3^{-/-}$ mice. Histologically, resting and growing oocytes in $Zp3^{-/-}$ females appeared normal and follicles of all stages, including post-ovulatory corpora lutea (evidence of past ovulation), were present within the ovary. However, in contrast to normal ovaries (Fig. 2A,B), there was a striking and complete absence of a zona pellucida matrix surrounding oocytes at all stages in $Zp3^{-/-}$ ovaries (Fig. 2C,D). Although the cells of the $Zp3^{-/-}$ corona radiata were closely adherent to the oocyte plasma membrane, they were consistently less uniformly arrayed than in normal animals (Fig. 2C,D). This observed disorganization of the corona radiata may reflect a need for zona matrix to maintain proper granulosa cell-oocyte interactions. However, even in the absence of the zona pellucida, oocytes grew in size and maintained intact germinal vesicles (nuclei), indicative of meiotic arrest. Similar to normal oocytes, $Zp3^{-/-}$ oocytes underwent germinal vesicle breakdown (GVBD) in the latter stages of folliculogenesis just prior to ovulation (Fig. 3). At the light microscopic level, the remainder of follicular architecture in $Zp3^{-/-}$ mice was indistinguishable from that of normal mice.

To investigate the effect of these abnormalities on later folliculogenesis, additional $Zp3^{+/+}$ and $Zp3^{-/-}$ females were primed with PMSG and killed 9 hours after the administration of hCG to accelerate the recruitment of follicles into the late growth phase without proceeding to ovulation. Comparable numbers of antral and preovulatory follicles were observed in normal and homozygous mutant mice. Oocytes were present within their antral follicles and the largest contained a cumulus oophorus and an oocyte that had undergone GVBD. In normal animals, the cumulus oophorus surrounding the oocyte was well structured with granulosa cells radiating out from a central oocyte (Fig. 3A,B). In contrast, the cumulus oophorus from $Zp3^{-/-}$ mice was poorly organized with granulosa cells randomly located in the cumulus oophorus (Fig. 3C,D). While the severity of this effect varied, in the extreme the cumulus oophorus was entirely separated from the zona-free oocyte (Fig. 3E,F). These data suggest that oocytes lacking a zona pellucida matrix develop during folliculogenesis but do not form a proper cumulus-oocyte complex. Thus, during the latter stages of folliculogenesis and ovulation, zona-free eggs are present but are free to dissociate from the cumulus mass.

Expression of *Zp1*, *Zp2* and *Zp3*

Although the disruption of the *Zp3* gene precluded the formation of the zona pellucida matrix, it did not prevent the expression of either *Zp1* or *Zp2*. Normal and mutant ovaries were embedded in paraffin and analyzed by in situ hybridization with ^{35}S-labeled anti-sense RNA probes specific to ZP1, ZP2 and ZP3. Each of the three zona transcripts was readily detected in normal ovaries (data not shown), where its expression was restricted to oocytes as previously reported (Epifano et al., 1995b). In mutant $Zp3^{-/-}$ ovaries, ZP3 transcripts were absent, but ZP1 and ZP2 transcripts were present in comparable amounts and with the same oocyte-restricted distribution as in the normal ovary (Fig. 4). Sense probes for each zona transcript gave no signal above background.

Similarly prepared sections were analyzed immunocytochemically with antibodies specific to each of the zona proteins (Epifano et al., 1995b; East and Dean, 1984; East et al., 1985). In $Zp3^{+/+}$ ovaries, ZP1, ZP2 and ZP3 were detected in the zona pellucida matrix surrounding growing oocytes as well as within the oocyte cytoplasm. In $Zp3^{-/-}$ ovaries, which lack zona

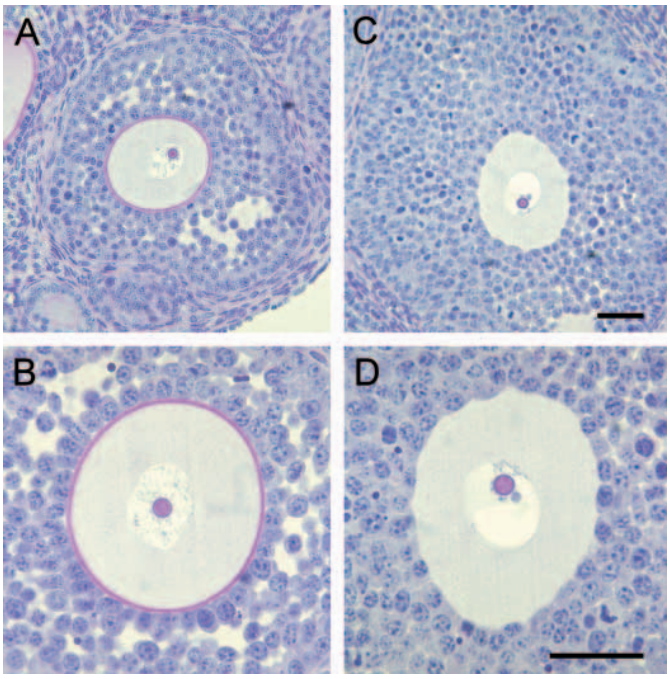

Fig. 2. Follicular histology of normal and homozygous mutant mice. (A) Pre-antral *Zp3*$^{+/+}$ follicle containing a 70-80 μm growing oocyte with an intact zona pellucida surrounded by multiple layers of granulosa cells. (B) Higher magnification of A showing the intact nucleus with a prominent nucleolus in the *Zp3*$^{+/+}$ follicle. The first layer of granulosa cells was well-organized into the corona radiata and was tightly adherent to the zona pellucida. (C) Pre-antral *Zp3*$^{-/-}$ follicle containing a 70-80 μm growing oocyte with an intact nucleus but no visible zona pellucida. (D) Higher magnification of C showing that in the absence of a zona pellucida the cells of the corona radiata were disorganized although still in close apposition to the oocyte. Scale bar, 40 μm.

matrices, ZP1 and ZP2 were present only within the oocyte cytoplasm and there was no immunolocalization with antibodies specific to ZP3 (data not shown). Individual growing oocytes were isolated from normal or *Zp3*$^{-/-}$ ovaries and stained with antibodies specific to each of the three zona proteins. Each of the three zona proteins was detected in the extracellular zonae pellucidae surrounding the normal oocytes (Fig. 5C,E,G). In *Zp3*$^{-/-}$ oocytes, which lack a zona pellucida, low levels of ZP1 and ZP2, but not ZP3, proteins were detected by confocal immunofluorescence microscopy at the surface of growing oocytes (Fig. 5D,F,G).

Fertility of *Zp3*$^{-/-}$ female mice

Fecundity was assessed by breeding normal, heterozygous and homozygous mutant mice with one another. When compared to normal matings, male mice, heterozygous or homozygous for the *Zp3* mutation, had normal fertility (Table 1). Likewise, female heterozygotes (*Zp3*$^{+/-}$) gave birth at the same time and with the same litter sizes as normal females. However, when mated with normal males, 8 female mice homozygous for the mutation (*Zp3*$^{-/-}$) did not become visibly pregnant and produced no litters. Normal females of identical ages were co-

Table 1. Number of live births

	Male parent		
Female parent	Normal (*Zp3*$^{+/+}$)	Heterozygous (*Zp3*$^{+/-}$)	Homozygous (*Zp3*$^{-/-}$)
Normal (*Zp3*$^{+/+}$)	7.5±0.6 (21)*	7.3±0.8 (11)	6.6±0.6 (13)
Heterozygous (*Zp3*$^{+/-}$)	9.1±0.8 (11)	7.8±0.5 (10)	9.3±1.2 (9)
Homozygous (*Zp3*$^{-/-}$)	0 (0)	n.m.†	n.m.

*Mean ± s.e. (number of litters) of 5 mating pairs except *Zp3*$^{-/-}$ female × *Zp3*$^{+/+}$ male and *Zp3*$^{+/+}$ female × *Zp3*$^{+/+}$ male crosses which used 8 and 9 mating pairs, respectively.
†Not mated.

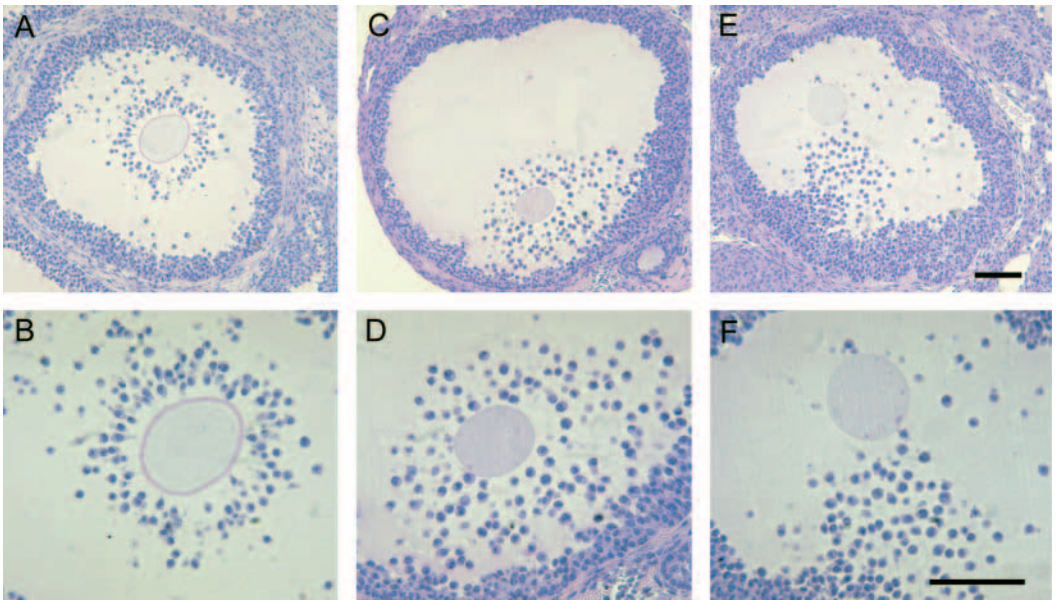

Fig. 3. Pre-ovulatory follicular histology of normal and homozygous mutant mice. (A) Normal metaphase II oocyte with an intact zona pellucida surrounded by a cumulus oophorus (cumulus granulosa cells in a matrix of hyaluronic acid) in the antrum of a pre-ovulatory *Zp3*$^{+/+}$ follicle. (B) Higher magnification of the *Zp3*$^{+/+}$ cumulus-oocyte complex in A detailing the absence of the nuclear membrane (germinal vesicle breakdown, GVBD), the acellular region between the intact zona pellucida and the cells of the concentrically expanded corona radiata, which appear tethered to the zona matrix by cellular processes. (C) Pre-ovulatory follicle in *Zp3*$^{-/-}$ mice that contained a GVBD oocyte lacking a zona pellucida. (D) Higher magnification of the *Zp3*$^{-/-}$ cumulus-oocyte complex in which the granulosa cells were disordered and, in the absence of the zona pellucida, were not concentrically arranged around the oocyte. (E) Pre-ovulatory *Zp3*$^{-/-}$ follicle with an even more disordered cumulus-oocyte complex. (F) Higher magnification of E showing the separation of the zona-free oocyte from the disorganized cumulus cells. The remaining follicular architecture appears normal. Scale bar, 100 μm.

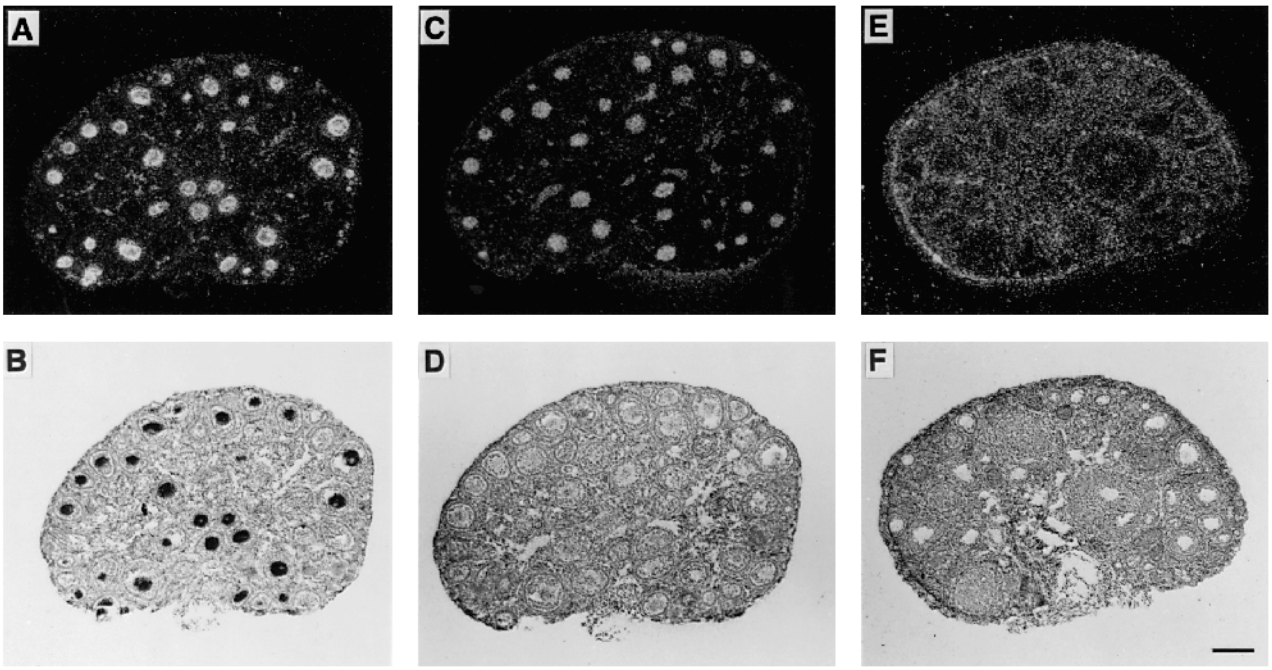

Fig. 4. In situ hybridization of $Zp3^{-/-}$ ovarian sections. ^{35}S-labeled anti-sense RNA probes derived from ZP1 (A,B), ZP2 (C,D) and ZP3 (E,F) cDNAs were hybridized to paraffin embedded ovarian sections and viewed under dark-field (A,C,E) and bright-field (B,D,F) optics. Probes specific to ZP1 (A,B) and ZP2 (C,D) but not ZP3 (E,F) hybridized to oocytes, within growing follicles throughout the ovary. Scale bar, 100 μm.

caged with the $Zp3^{-/-}$ females and their repeated ability to give birth to litters demonstrated the continued fertility of the normal male.

To assess whether ovulation could occur in the homozygous mutant mice, 4- to 6-week old female mice (8 $Zp3^{+/+}$; 13 $Zp3^{-/-}$) were primed with pregnant mare serum gonadotrophin (PMSG). 20 hours after administration of human chorionic gonadotrophin (hCG) to induce ovulation, the females were killed and their oviducts were excised for collection of oocytes. All of the control animals had cumulus masses in each oviduct, which contained an average of 31.1±2.4 ovulated eggs per animal (Table 2). Cumulus masses, albeit smaller, were also recovered from all the $Zp3^{-/-}$ females. However, eggs were detected in only 6 of the 13 $Zp3^{-/-}$ mice and these animals had many fewer eggs (1-5 eggs; mean, 2.3±0.7).

Zona-free eggs can be fertilized in vitro by spontaneously acrosome-reacted sperm (Pavlok and McLaren, 1972; Naito et al., 1992). To determine if the eggs ovulated by $Zp3^{-/-}$ mice could be fertilized in vivo and recovered as 2-cell embryos, normal and homozygous females were induced hormonally to ovulate and mated with males proven to be fertile. Using the presence of vaginal plugs as evidence of copulation, 5 normal and 7 homozygous mutant female mice were killed 40 hours after the administration of hCG and their oviducts were flushed with saline. An average of 17.5±3.2 2-cell embryos were recovered from the normal females, but no 2-cell embryos or eggs were recovered from the homozygous mutant $Zp3^{-/-}$ mice (Table 2). Thus, although preovulatory GVBD oocytes are present in the ovary, the number of zona-free eggs that can be recovered from mutant $Zp3^{-/-}$ mice after ovulation is very small and no fertilized embryos have been recovered.

Table 2. Number of ovulated eggs or 2-cell embryos

	Ovulated eggs	2-cell embryos
Normal ($Zp3^{+/+}$)	31.1±2.4 (8/8)*	17.5±3.5 (5/5)†
Homozygous ($Zp3^{-/-}$)	2.3±0.7 (6/13)	0 (0/7)

*Mean ± s.e. (number of positive animals/total number of animals).
†Mean ± s.e. (number of positive animals/total number of plugged animals).

DISCUSSION

Although abnormal, folliculogenesis in the homozygous mutant mice supports the development of the oocyte through GVBD and subsequent ovulation. However, few zona-free $Zp3^{-/-}$ eggs are observed in flushed oviducts 16 hours after hCG stimulation and no 2-cell embryos are observed 40 hours after hCG stimulation in $Zp3^{-/-}$ females mated with fertile males. Thus, homozygous mutant $Zp3^{-/-}$ females do not become visibly pregnant and none has produced litters. Normal eggs, chemically freed from zonae pellucidae, can be fertilized in vitro and developed to the blastocyst stage. When these blastocysts are transferred to pseudopregnant foster mothers, live births are obtained (Naito et al., 1992). These results indicate that the absence of a zona pellucida does not preclude fertilization, at least in vitro. However, if 1- to 4-cell, zona-free embryos are surgically transferred to oviducts, no live births are observed and no embryos are recovered from oviducts flushed more than 2 hours after transfer. Histological examination reveals adherence of the zona-free embryos to the epithelium of the oviduct within several hours of transfer. No cleavage occurs in the immobilized embryos and all embryos

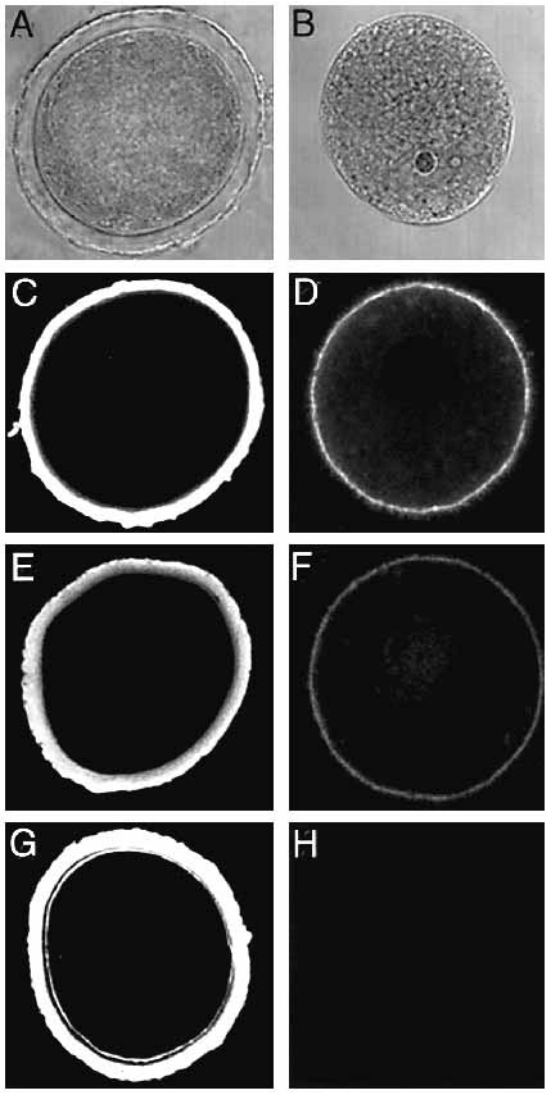

Fig. 5. Immunocytochemistry of isolated oocytes. Mid-sized oocyte from a 3-week old *Zp3*$^{+/+}$ mouse with an intact zona pellucida (A) and a *Zp3*$^{-/-}$ mouse that lacks a zona pellucida (B) were viewed by phase contrast microscopy. Confocal microscopy of *Zp3*$^{+/+}$ oocytes stained with antibodies specific to ZP1 (C), ZP2 (E) and ZP3 (G) localized each of the three zona proteins to the extracellular zona pellucida matrix. Confocal microscopy of *Zp3*$^{-/-}$ oocytes stained with antibodies specific to ZP1 (D), ZP2 (F) and ZP3 (H), localized ZP1 and ZP2, but not ZP3, to the surface of the oocyte. Similar results were obtained with or without saponin to permeabilize the membrane.

disappear within 24 hours of transfer (Bronson and McLaren, 1970; Modlinski, 1970). The infertile phenotype of the *Zp3*$^{-/-}$ female mice most likely reflects a similar adherence of the zona-free egg to the oviductal epithelium, and zona-free *Zp3*$^{-/-}$ eggs, even if fertilized, would not cleave or pass down the oviduct. However, it remains important to assess the developmental competence of zona-free null eggs by transferring them to pseudopregnant females after in vitro fertilization, a task complicated by the few ovulated eggs (approximately one per animal) that can be retrieved from *Zp3*$^{-/-}$ females.

It is surprising that a genetic defect causing this striking phenotype has not been observed in other mammals, particularly humans who seek medical attention for infertility and whose therapy may include the retrieval of eggs. Humans, like all mammals, have a zona pellucida that surrounds oocytes, eggs and early embryos. The human *ZP1*, *ZP2*, and *ZP3* genes are conserved and each encodes a zona protein with an amino acid sequence that is 42%, 61% and 67% identical, respectively, to their murine orthologues (Chamberlin and Dean, 1990; Liang and Dean, 1993; Harris et al., 1994). Since the first in vitro fertilization resulting in a live human birth (Steptoe and Edwards, 1978), eggs from hundreds of thousands of women have been retrieved. As yet, none has been reported to lack a zona pellucida. Thus, although the murine phenotype demonstrates that the absence of a zona pellucida causes infertility, it must be a relatively rare cause of infertility in humans. However, the lack of a zona pellucida could render human oocytes too fragile to be successfully retrieved by current techniques and hence examination of women from whom oocyte retrieval is unsuccessful might reveal a *ZP3*$^{-/-}$ genotype.

Despite the absence of a zona matrix in *Zp3*$^{-/-}$ mice, folliculogenesis occurs and follicles of all stages are present in the ovary. However, the layer of granulosa cells closest to the oocyte that form the corona radiata is disorganized in *Zp3*$^{-/-}$ ovaries compared to normal mice. It is this layer of granulosa cells that normally develop processes to penetrate through the zona pellucida and form gap junctions with the oocyte (Anderson and Albertini, 1976; Gilula et al., 1978). This contact is reportedly essential for oocyte growth (Eppig, 1979; Heller et al., 1981) and inhibition of premature meiotic maturation (Schultz and Wassarman, 1977). In spite of the perturbation of the corona radiata, the zona-free oocytes grow normally during folliculogenesis with no evidence of premature progression of meiosis, suggesting that essential oocyte-granulosa cell interactions remain intact.

In normal mice, the preovulatory surge of gonadotropins disrupts the gap-junction mediated syncytium of mural and cumulus granulosa cells with the oocyte. The oocyte undergoes GVBD to complete the first meiotic division and arrests at metaphase II. Secretion of hyaluronic acid by the cumulus cells results in an expanded cumulus oophorus in which oocytes and cumulus cells are enveloped in a mucinous cumulus mass (Eppig, 1991). Pre-ovulatory oocytes in *Zp3*$^{-/-}$ females also undergo GVBD, but the absence of a zona pellucida matrix results in a disorganized cumulus oophorus. The acellular region normally separating the cumulus cells from the oocyte is absent and the cumulus oophorus lacks a distinct corona radiata. Instead, the granulosa cells are randomly arrayed in the cumulus mass and, not infrequently, the oocyte is separate from the cumulus oophorus. The absence of the zona matrix in which residual cellular processes tether the cells of the corona radiata could account for the disorganization of the cumulus oophorus in the *Zp3*$^{-/-}$ pre-ovulatory follicle. Within 16-20 hours of the gonadotrophin surge in normal mice, ovulated eggs are present in the oviduct still embedded in the cumulus mass. Although cumulus masses are also observed in *Zp3*$^{-/-}$ mice, less than half have eggs associated with the cumulus matrix and those that do have many fewer (<10% of normal) eggs.

The current results demonstrate that the formation of the zona pellucida matrix is precluded by the absence of a single zona protein, ZP3. An earlier in vitro transient assay established that

anti-sense oligonucleotides injected into the cytoplasm of growing oocytes could specifically degrade either ZP2 or ZP3 mRNA (Tong et al., 1995). Within the oocyte, de novo biosynthesis of the zona protein corresponding to the targeted mRNA was inhibited, but the biosynthesis of the other zona protein was unaffected. Although neither protein was incorporated into a pre-existing zona pellucida matrix, the effect of the absent zona protein on initial zona matrix formation during oogenesis could not be studied. We now extend these earlier observations in vivo and demonstrate that in $Zp3^{-/-}$ mice, the absence of ZP3 does not prevent accumulation of ZP1 or ZP2 transcripts or translation into their cognate proteins. However, no zona pellucida matrix is formed. Taken together, these data suggest that the biosynthesis of each zona protein occurs independently, but their formation into a zona matrix requires the presence of all three proteins.

Little is known about the mechanisms by which the zona matrix is formed. The primary structures of the three mouse zona proteins have been deduced from full-length cDNAs (Ringuette et al., 1988; Liang et al., 1990; Epifano et al., 1995b). Each has a signal peptide to direct it into a secretory pathway. Each has a hydrophobic region (22-26 amino acids) near its carboxyl terminus that is preceded (31-47 amino acids) by a potential endoprotease cleavage site (Yurewicz et al., 1993). The detection of low levels of ZP1 and ZP2 proteins at the oocyte surface in zona-free $Zp3^{-/-}$ mice raises the possibility that this hydrophobic region acts as a membrane spanning domain, anchoring the zona proteins in the plasma membrane, and that subsequent endoproteolytic cleavage facilitates the integration of the zona proteins into the extracellular matrix surrounding the oocyte. Repetitive release of zona proteins from the oocyte surface could account for the growth of the zona matrix during oogenesis. The oocyte-surface localization of ZP1 and ZP2 is consistent with this model and the rescue of the homozygous mutant $Zp3^{-/-}$ mice with normal ZP3 and ZP3 mutated at the potential proteolytic processing site will be useful in investigating the mechanisms of zona matrix formation.

We would like to warmly acknowledge the contributions to earlier aspects of these studies by Dr D. R. Lunsford. We thank Dr J. Jaenisch for the J1 cells, Dr Prue Talbot for useful discussions on microscopy and Drs T. Quick and L. Henninghausen for critical reading of the manuscript. Animal care was provided according to NIH guidelines.

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(Accepted 21 June 1996)

Note added in press

While this manuscript was under review, the following paper was published: **Liu, C., Litscher, E. S., Mortillo, S., Sakai, Y. Kinloch, R. A., Stewart, C. L. and Wassarman, P. M.** (1996). Targeted disruption of the mZP3 gene results in production of eggs lacking a zona pellucida and infertily in female mice, *Proc. Natl. Acad. Sci, USA* **93**, 5431-5436.