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Defective zonae pellucidae in *Zp2*-null mice disrupt folliculogenesis, fertility and development

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SUMMARY

All vertebrate eggs are surrounded by an extracellular matrix. This matrix is known as the zona pellucida in mammals and is critically important for the survival of growing oocytes, successful fertilization and the passage of early embryos through the oviduct. The mouse zona pellucida is composed of three glycoproteins (ZP1, ZP2 and ZP3), each encoded by a single copy gene. Using targeted mutagenesis in embryonic stem cells, *Zp2*-null mouse lines have been established. ZP1 and ZP3 proteins continue to be synthesized and form a thin zona matrix in early follicles that is not sustained in pre-ovulatory follicles. The abnormal zona matrix does not affect initial folliculogenesis, but there is a significant decrease in the number of antral stage follicles in ovaries isolated from mice lacking a zona pellucida. Few eggs are detected in the oviduct after stimulation with gonadotropins, and no two-cell embryos are recovered after mating *Zp2*-null females with normal male mice. The structural defect is more severe than that observed in *Zp1*-null mice, which have decreased fecundity, but not quite as severe as that observed in *Zp3*-null mice, which never form a visible zona pellucida and are sterile. Although zona-free oocytes matured and fertilized in vitro can progress to the blastocyst stage, the developmental potential of blastocysts derived from either *Zp2*- or *Zp3*-null eggs appears compromised and, after transfer to foster mothers, live births have not been observed. Thus, in addition to its role in fertilization and protection of early embryos, these data are consistent with the zona pellucida maintaining interactions between granulosa cells and oocytes during folliculogenesis that are critical to maximize developmental competence of oocytes.

Key words: Zona pellucida, Fertilization, Embryonic stem cells, Sperm-egg interactions, Transgenic mice, Folliculogenesis, Pre-implantation development, ZP2, Oocyte-granulosa cell interactions

INTRODUCTION

The mouse zona pellucida, composed of three glycoproteins (ZP1, ZP2 and ZP3), is an extracellular matrix that surrounds growing oocytes, ovulated eggs and early embryos (for review, see Rankin and Dean, 2000). Perinatally, primordial follicles are formed in which oocytes, arrested in the prophase of the first meiotic division, are enclosed in a layer of squamous granulosa cells surrounded by a basement membrane (Brambell, 1928). These primordial follicles (17 μm) either persist during the functional life of the ovary or begin to grow in response to, as yet, poorly defined signal(s). At the onset of folliculogenesis, the flattened granulosa cells of the primordial follicle become cuboidal in primary follicles and subsequent proliferation results in secondary follicles with two to three layers of granulosa cells. Later in folliculogenesis, a fluid-filled antrum forms as the follicle reaches 180-200 μm and enlarges as the follicle attains its full size (400-600 μm) just prior to ovulation. During the initial two weeks of folliculogenesis, oocytes grow from 12-15 μm to 80 μm (in the early antral follicle), but grow little thereafter. Although meiotically competent at this stage, the oocyte is held in arrest by interactions with the granulosa cells until the preovulatory follicle stage, at which time it progresses to metaphase II in anticipation of ovulation and subsequent fertilization.

The zona pellucida is first observed as extracellular patches that coalesce into a uniform matrix surrounding oocytes in primary follicles and increases in width to 7 μm surrounding fully grown oocytes in the early antral follicles (Odor and Blandau, 1969; Dietl, 1989). Although the zona matrix physically separates the oocyte and somatic cells, close associations are maintained throughout follicular development via paracrine factors and cellular processes that penetrate the zona pellucida to form gap junctions (Eppig, 1991). *Zp1*-null mice form a zona pellucida with only ZP2 and ZP3, but the matrix is structurally flawed and approximately ten percent of growing follicles have ectopic granulosa cells in the perivitelline space between the oolemma and zona pellucida. Later in folliculogenesis, some *Zp1*-null follicles develop accentuated perivitelline spaces prior to ovulation. Although *Zp1*-null females are fertile, they have decreased fecundity because of precocious hatching of early embryos from the

structurally compromised zona matrix (Rankin et al., 1999). A more dramatic phenotype is observed in mice that lack ZP3, which are unable to form a zona matrix despite synthesis of ZP1 and ZP2. Although some eggs are recovered from *Zp3*-null mice after gonadotropin stimulation, two-cell embryos are not observed in the oviduct after mating and females are sterile (Liu et al., 1996; Rankin et al., 1996).

It was unclear whether the absence of ZP2 would result in a phenotype similar to mice lacking ZP1 or ZP3. During oogenesis, ZP2 and ZP3 transcripts are present in approximately stoichometric amounts, with ZP1 being less abundant (Epifano et al., 1995b). A model of the zona pellucida matrix proposes that ZP2/ZP3 protein dimers form a meshwork of fibrils that are periodically crosslinked by homodimers of ZP1 (Greve and Wassarman, 1985; Green, 1997). These observations suggest that the absence of ZP2 would phenocopy *Zp3*-null mice and result in zona-free oocytes and sterility. However, the ~350 amino acid domain at the C terminus of ZP2 (713 amino acids) is 47% similar to ZP1 (623 amino acids; Epifano et al., 1995a), which raises the possibility that ZP1 might substitute for ZP2 in forming a zona matrix in *Zp2*-null mice. If true, then the absence of ZP2 might produce a similar phenotype to that of *Zp1*-null mice, in which a zona pellucida is formed, but structural abnormalities lead to precocious hatching and decreased fecundity. To determine experimentally the consequence of absent ZP2 and further investigate the biological functions of the zona pellucida, we have established mouse lines that lack ZP2 and compared the effects of each (*Zp1*, *Zp2* and *Zp3*) null mutation on folliculogenesis, fertilization and early development.

MATERIALS AND METHODS

Gene targeting

The targeting vector was constructed using a genomic clone of mouse *Zp2* obtained from screening a 129/Sv mouse genomic library (Stratagene, La Jolla CA) with mouse ZP2 cDNA (Liang et al., 1990). A 1.2 kb *Pme*I-*Kpn*I fragment containing a portion of the *Zp2* promoter and exon 1 (including the ATG initiation site) was subcloned into the *Xho*I site of pPNT and a second, 5 kb *Eco*RI-*Spe*I fragment containing exons 3-7, was cloned into the *Xba*I site (Fig. 1). The targeting construct was linearized with *Not*I and electroporated into R1 embryonic stem cells (Nagy et al., 1993). Individual clones were selected after growth in G418 (Life Technologies, Rockville, MD) and gangcyclovir (Roche Discovery, Welwyn, UK), and correctly targeted clones were identified by Southern blot analysis with ^{32}P-labeled probes 5′ (−1570 to −1277 bp of the transcription start site) and 3′ (cDNA encoding exons 11-14) to the targeting vector (Fig. 1). Identity of the mutant allele was confirmed using a 0.6 kb *Pst*I fragment isolated from PGK-*Neo*. Heterozygous *Zp2*$^{tm/+}$ cells from multiple lines were injected into C57BL/6N blastocysts to obtain coat color chimeric mice. Germ cell transmission was assayed by Southern blot analysis of DNA isolated from tails (Rankin et al., 1996).

Ovarian histology

Ovaries were isolated from 6-8-week-old normal or *Zp2*-null females, fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 overnight, rinsed in the same and transferred to 70% ethanol. Tissues were dehydrated, embedded in methacrylate and 2 μm sections cut (American Histolabs, Gaithersburg, MD). Mounted sections were stained with periodic-acid Schiff's reagent and Hematoxylin. Additional mice were stimulated with 5 iu each of pregnant mare serum gonadotrophins (eCG; Sigma, St Louis, MO) administered intraperitoneally, followed 48 hours later with 5 iu each of human chorionic gonadotrophin (hCG; Sigma, St Louis, MO). Nine hours after hCG administration, the ovaries were isolated, fixed and sectioned as described above to observe preovulatory follicles. Alternatively, single ovaries (four to seven) were isolated from 21-day-old normal and *Zp1*-, *Zp2*- and *Zp3*-null mice and fixed overnight in Bouin's (Rankin et al., 1996) for morphometric analysis of folliculogenesis (Pedersen and Peters, 1968). The number of primary, preantral, early antral and mid-to-large antral follicles was estimated by counting follicles, in every third section in which the germinal vesicle of the oocyte was observed. Comparisons of null mutants with normal was performed by one-way ANOVA with Dunnett's post test using GraphPad InStat version 3.02 for Windows 95 (GraphPad Software, San Diego, CA).

Isolation of eggs and embryos

Normal and *Zp2*-null females were stimulated with 5 iu each of eCG followed by 5 iu each of hCG 46-48 hours later. For collection of ovulated eggs, the mice were euthanized 14 hours after administration of hCG and cumulus masses were isolated from oviducts and dispersed with hyaluronidase Type IV (0.5 mg/ml; Sigma, St Louis, MO) for 2-3 minutes. To obtain two-cell embryos, mice were mated with (CBA/N × C57BL/6N)F_1 males at the time of hCG administration and plugged females were euthanized 40 hours later. Embryos were collected by flushing the oviducts with M2 medium (Specialty Media, Lavallette, NJ).

Immunocytochemistry and confocal microscopy

Ovaries were isolated from 21-day-old normal, *Zp1*- (Rankin et al., 1999), *Zp2*- and *Zp3*- (Rankin et al., 1996) null mice, fixed for 5 hours at room temperature in 2% paraformaldehyde in phosphate-buffered saline (PBS; 127 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4), dehydrated through graded alcohol solutions, and embedded in paraffin (American Histolabs, Gaithersburg, MD). Sections (4-6 μm) were cut, rehydrated, rinsed in Tris-buffered saline (TBS; 10 mM Tris-HCl, pH 7.6, 0.15 mM NaCl) and blocked with 3% BSA in TBS (BSA/TBS) for 1 hour at room temperature. Sections were incubated (overnight, 4ºC) with antibodies to ZP1 (Rankin et al., 1998), ZP2 (East and Dean, 1984) or ZP3 (East et al., 1985) diluted (1:500) in BSA/TBS. After washing the slides three times (3 minutes each wash) in BSA/TBS, the slides stained with antibody to ZP1 were incubated (1 hour, room temperature) with AMCA-conjugated goat anti-rat IgG; the slides stained with antibody to ZP2 were incubated with TRITC-conjugated goat anti-rat IgG; and the slides stained with antibody to ZP3 were incubated with FITC-conjugated goat anti-rat IgG (all from Jackson Immunoresearch, West Grove, PA). At the end of the incubation, the slides were washed three times (3 minutes each wash) with BSA/TBS and equilibrated in Slow-Fade Light buffer before mounting in Slow-Fade Light mounting medium (Molecular Probes, Eugene, OR). Imaging was performed on a Zeis Axiovert 100M microscope equipped with a LSM 510 laser-scanning confocal optics (Carl Zeiss, Thornwood, NY). For imaging AMCA emissions, excitation filters of 364 nm and 488 nm, and a 385 nm LP emission filter were used; for rhodamine emissions, excitation filters of 543 nm and 488 nm and a 560 nm LP filter were used; and for FITC, an excitation filter of 488 nm and a 505 nm LP filter were used. Differential interference microscopy images were taken on the same system using a single channel transmission detector. Settings were optimized for visualizing the normal zona pellucida and those settings were used to image the zonae from each of the three null lines.

Isolation and culture of oocytes and preimplantation embryos

Germinal vesicle stage oocytes were isolated from antral follicles of eCG-primed mice and matured in vitro (Eppig and O'Brien, 1996). After 15 hours of maturation, eggs were fertilized in vitro and zygotes

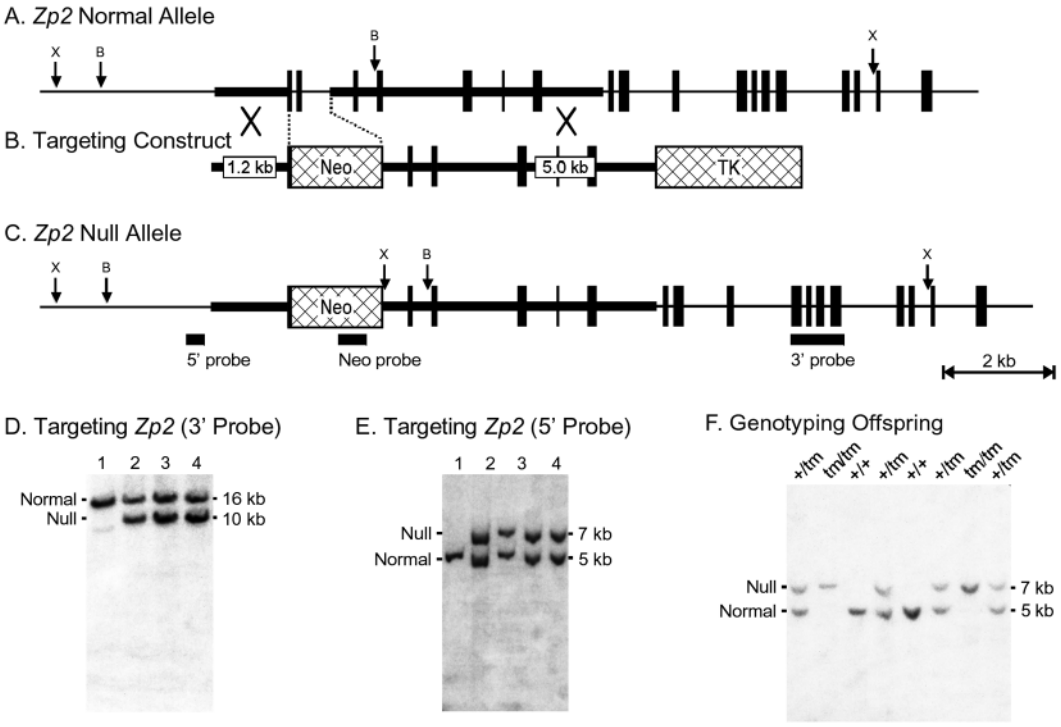

Fig. 1. Targeting the *Zp2* locus in embryonic stem cells. (A) The *Zp2* allele containing 18 exons on chromosome 7. (B) The targeting construct generated by inserting the PGK-Neo and the PGK-TK cassettes as positive and negative selection markers. (C) The resulting null allele generated by the homologous replacement of the normal allele with the targeting construct. Vertical bars represent exons and arrows indicate restriction endonuclease sites. The thicker lines represent the 1.2 kb and 5.0 kb of homologous DNA that are 5′ and 3′, respectively, to the Neo cassette in the targeting construct. The probes used for Southern hybridization are indicated under the targeted allele (C) by black boxes. X, *Xba*I; B, *Bam*HI. Southern hybridization of purified embryonic stem cell DNA using probes 3′ (D) and 5′ (E) of the recombination breakpoints. After digestion with *Xba*I, the normal and mutant alleles detected with the 3′ probe had fragment sizes of 16 kb and 10 kb, respectively. After digestion with *Bam*HI, the normal and mutant alleles detected with the 5′ probe had sizes of 5 kb and 7 kb, respectively. Lanes 1 contain the DNA of untargeted embryonic stem cells and lanes 2-4 contain the DNA of successfully targeted stem cells. (F) Genotyping by Southern blot hybridization of DNA isolated from the tails of F_2 animals generated from a $Zp2^{+/tm} \times Zp2^{+/tm}$ cross after restriction digest with *Bam*HI and hybridization with the 5′ probe. Normal $Zp2^{+/+}$; heterozygous $Zp2^{+/tm}$ and homozygous $Zp2^{tm/tm}$ animals were present in the expected Mendelian ratios for transmission of a single copy mutant gene.

were cultured as described (Eppig and O'Brien, 1996), except that embryos were placed in individual drops (20 μl) of medium under washed paraffin oil to prevent zona-free embryos from adhering to one another. The zonae pellucidae were removed from normal oocytes by brief exposure to acidified Tyrode's media (Hogan et al., 1994) and decreased quantities of sperm (2.5×10^3) were used to fertilize eggs lacking a zona pellucida. After in vitro culture, blastocysts were transferred to pseudopregnant female mice (Eppig and O'Brien, 1996).

RESULTS

Targeting the *Zp2* gene

The mouse *Zp2* gene (Liang et al., 1990) was re-isolated from a 129/Sv genomic library and used to construct a targeting vector of the single-copy gene located on chromosome 7 (Fig. 1A, Lunsford et al., 1990). The construct contained 6.2 kb of isogenic DNA (1.2 kb 5′ to the transcription start site and 5.0 kb extending from intron 2 to intron 7, Fig. 1B). After electroporation into R1 embryonic stem cells and positive-negative section with G418 and gangcyclovir (Thomas and Capecchi, 1987), 6% of the surviving cells were successfully targeted (Fig. 1C). Targeting was confirmed using 5′ and 3′ probes that lay outside the range of homologous DNA (Fig. 1D,E). Independently derived heterozygous null cell lines were injected into C57BL/6 host blastocysts to establish two mouse lines. Coat color chimeras were bred to CF-1 females to assure germline transmission of mutant *Zp2* and obtain F_1 heterozygotes. Male and female heterozygotes were crossed and produced $Zp2^{+/+}$, $Zp2^{+/tm}$, and $Zp2^{tm/tm}$ offspring in the expected Mendelian ratios of a single-copy mutant gene (Fig. 1F).

The absence of ZP2 protein in *Zp2*-null mice was confirmed in paraffin-embedded ovarian sections (data not shown) and by confocal microscopy (Fig. 3H) using a monoclonal antibody specific to ZP2 that recognizes a linear epitope (amino acids 114-129) encoded by exon 5 (Greenhouse et al., 1999). Two independently derived mouse lines ($Zp2^{tm1Nih/tm1Nih}$ and $Zp2^{tm2Nih/tm2Nih}$) with germline transmission have been stably maintained for over 2 years.

Ovarian histology of *Zp2*-null mice

The external morphology and behavior of both male and female *Zp2*-null mice appeared normal, and the ovaries of *Zp2*-null females were grossly indistinguishable from normal littermates. When *Zp2*-null ovaries were examined

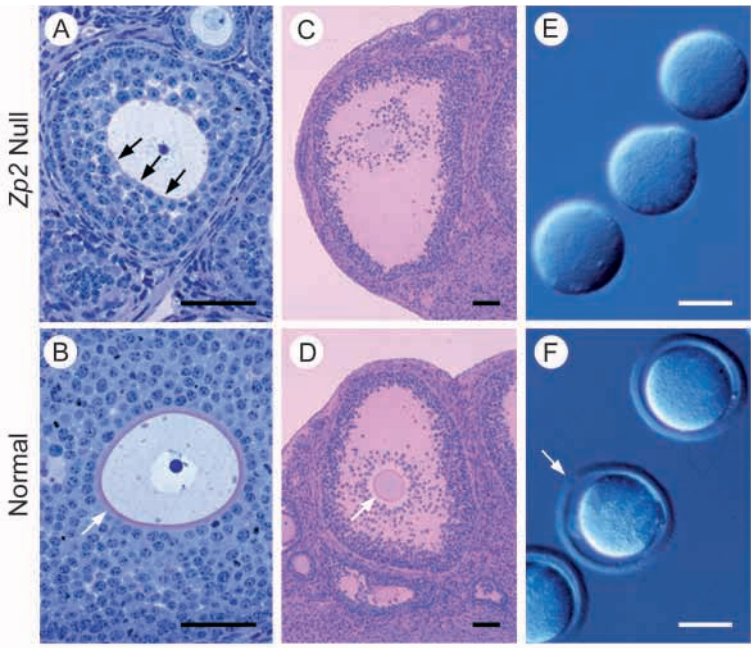

Fig. 2. Ovarian histology and eggs from *Zp2*-null females. Ovaries isolated from both *Zp2*-null (A,C) and normal (B,D) mice, either in the absence of gonadotropin stimulation (A,B) or 9 hours post-hCG (C,D) were fixed in 3% glutaraldehyde and stained with periodic-acid Schiff's (PAS) reagent and Hematoxylin to highlight the glycoprotein-rich zona pellucida. *Zp2*-null females have a very thin zona pellucida (A, arrows) compared with normal (B, arrow). The thin zona pellucida does not persist after formation of the antrum (C) of null mice, but is clearly present as a PAS-stained structure surrounding normal oocytes (D, arrow). In these preovulatory follicles, the oophorus-cumulus complexes in the zona-free *Zp2*-null females (C) are highly disorganized compared with normal complexes (D). The *Zp2*-null oocytes have resumed meiosis, shown by the absence of a germinal vesicle (nucleus), indicating normal meiotic maturation in the zona-free oocytes. Isolated eggs were collected from the oviducts of *Zp2*-null (E) and normal females (F). The eggs isolated from the *Zp2*-null females completely lacked a zona matrix which was clearly visible surrounding normal ovulated eggs (F, arrow). Scale bars: 50 μm.

histologically, follicles of all stages were present, including corpora lutea resulting from past ovulations. During pre-antral folliculogenesis, a very thin zona pellucida was present in the perivitelline space between the plasma membrane of the oocytes and the surrounding granulosa cells (Fig. 2A, arrows). However, this nascent zona matrix was not sustained beyond the antral stage and zonae pellucidae were not observed late in folliculogenesis (Fig. 2C) or in ovulated eggs (Fig. 2E). Despite the severe defects in their zonae pellucidae, oocytes grew in size, remained in meiotic arrest and the rest of the follicular architecture was morphologically unperturbed in the *Zp2*-null follicles.

Later in folliculogenesis, however, the absence of a zona matrix resulted in dramatic alteration of somatic-oocyte interactions. Normally in pre-ovulatory follicles during the mucification reaction, the cumulus (granulosa) cells surrounding the egg secrete hyaluronic acid which separates the cells from one another and from the centrally positioned germ cell. However, the somatic cells remain tethered to the zona matrix by cellular processes and establish a distinctive oocyte- cumulus complex (Fig. 2D). In the absence of the zona pellucida, the cumulus cells are not regularly arrayed around the central germ cell and the oocyte-cumulus complex becomes progressively disorganized (Fig. 2C). Nevertheless, the zona-free oocytes undergo germinal vesicle breakdown (Fig. 2C) and can be ovulated into the oviduct (Fig. 2E).

Fertility of *Zp2*-null mice

The fertility of the mice was assessed by breeding *Zp2*-null and normal mice. As anticipated, *Zp2*-null male mice were as fertile as normal cagemates (data not shown). However, female *Zp2*-null mice never became visibly pregnant and produced no offspring when mated with normal males (Table 1). To determine the cause of this infertility, *Zp2*-null and normal mice were stimulated with gonadotropins to obtain ovulated eggs. Both the *Zp2*-null and normal mice responded to hormonal stimulation and cumulus masses were isolated from their oviducts. However, ovulated eggs were isolated from only half (6/12) of the *Zp2*-null mice and the number of eggs (1.4±0.6) was less than 5% of those obtained from normal females (35.6±7.7). When *Zp2*-null females were mated and their oviducts were flushed for two-cell embryos 40 hours after hCG stimulation, none was found (Table 1). Thus, the absence of a zona pellucida precludes formation of a normal oophorus-cumulus complex and very few eggs are present in the oviducts of null females. Additionally, any zona-free embryos resulting from the fertilization of these eggs failed to survive to the two-cell stage.

Expression of zona pellucida proteins in *Zp1*-, *Zp2*- and *Zp3*-null follicles

To examine the accumulation of zona pellucida proteins in null animals, ovaries were isolated from normal and mutant 21 day old females and stained with monoclonal antibodies specific to ZP1, ZP2 or ZP3. All three zona pellucida proteins were detected in the zona pellucida surrounding normal growing oocytes by confocal microscopy (Fig. 3A-C) and the same settings were used to image the null ovaries. As anticipated, the zona pellucida protein encoded by the disrupted gene was not detected in *Zp1*-, *Zp2*- and *Zp3*-null ovaries (Fig. 3D,H,L). In the absence of ZP1, both ZP2 and ZP3 were incorporated into a zona matrix and the observed signal was comparable with that seen in the normal ovary (Fig. 3D-F). In the absence of ZP2, both ZP1 and ZP3 were detected at the periphery of

Table 1. Fertility of homozygous *Zp2*-null female mice

	Ovulated eggs*	Two-cell embryos*	Live births‡
Normal ($Zp2^{+/+}$)	35.6±7.7 (8/8)	17.6±6.1 (8)	8.6±0.6 (31)
Homozygous null ($Zp2^{tm/tm}$)	1.4±0.6 (6/12)	0 (7)	0 (0)

*Average±s.e.m. (number of animals from which eggs recovered/number of animals).
‡Average±s.e.m. (number of litters).

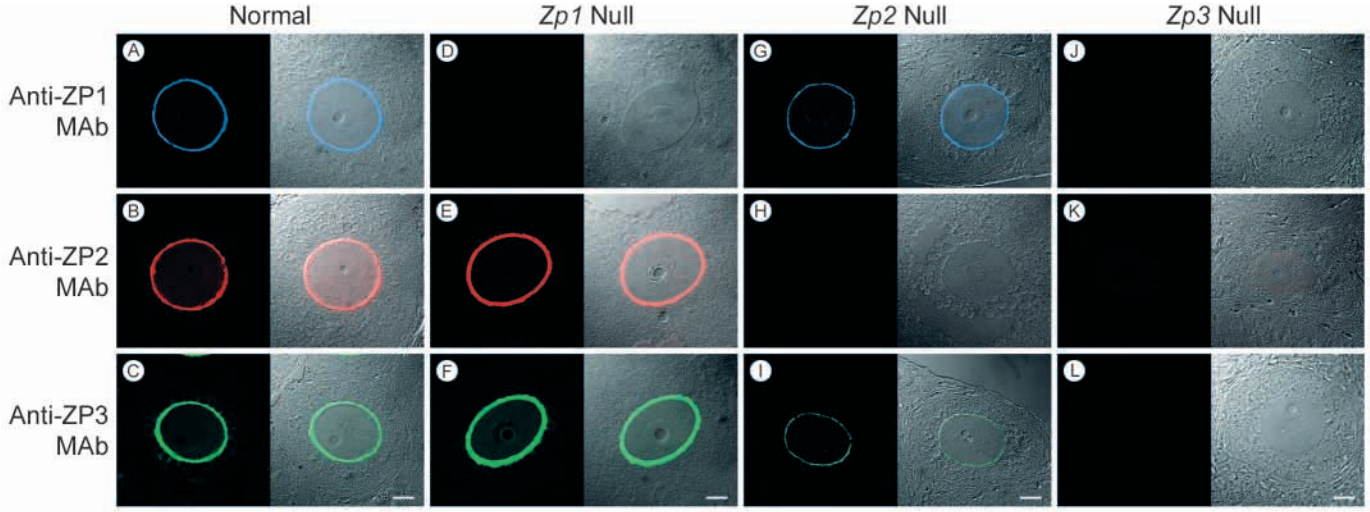

Fig. 3. Confocal microscopy of ovarian sections isolated from Zp-null mice. Ovaries were isolated from normal (A-C), *Zp1*- (D-F), *Zp2*- (G-I) and *Zp3*- (J-L) null females, fixed, sectioned and stained with monoclonal antibodies (MAb) to ZP1 (A,D,G,J), ZP2 (B,E,H,K) and ZP3 (C,F,I,L). In normal follicles, ZP1, ZP2 and ZP3 were present in the zona pellucida surrounding growing oocyte. *Zp1*-null oocytes had a zona matrix composed of ZP2 and ZP3 (E,F), and the *Zp2*-null oocytes had a zona matrix composed of ZP1 and ZP3 (G,I). However, the zona pellucida surrounding *Zp2* oocytes was considerably thinner than that surrounding *Zp1*-null oocytes. No zona matrix was observed surrounding *Zp3*-null oocytes (J-L). Images of the null ovaries with each MAb were obtained with the same settings as were used for the normal ovary. Scale bars: 20 μm.

oocytes, consistent with co-localization with the thin zona matrix observed by light microscopy (Fig. 2A, arrows; Fig. 3G-I). However, the intensity of the confocal images, particularly of ZP3, was significantly less than that observed in the normal ovary. As has been reported earlier, the absence of ZP3 appeared to preclude the formation of a visible extracellular zona matrix (Fig. 3J-L), although both ZP1 and ZP2 were detected in the oocyte using greater gain on the confocal microscope (data not shown).

Thus, the phenotype of the *Zp2*-null seems to be intermediate between *Zp1*-null mice, which have an abnormal, but clearly present zona matrix, and *Zp3*-null mice, which have no zona pellucida. It is not clear whether the thinness of the zona matrix in *Zp2*-null mice reflects a stoichometric limitation of ZP1 compared with the considerably more abundant ZP2, or whether it reflects a less robust interaction between ZP1 and ZP3 compared with that between ZP2 and ZP3. Taken together, these data reinforce earlier observations from the *Zp1*- (Rankin et al., 1999) and the *Zp3*- (Rankin et al., 1996) null mice that the zona pellucida proteins are synthesized independently from each other, even though their transcription is coordinately regulated (Epifano et al., 1995b). It remains unclear, however, how or where (intraorganelle, plasma surface, perivitelline space) the zona matrix is assembled during oogenesis.

Folliculogenesis in females lacking individual zona pellucida proteins

Although follicles of all stages were present in the ovaries of females lacking either ZP1, ZP2 or ZP3, it was of interest to examine further the dynamics of folliculogenesis in these animals. To that end, four to seven ovaries from normal and each of the lines (21 day old) were isolated, fixed and serially sectioned for morphometric analysis.

Normally, as folliculogenesis progresses from primary to antral stages, the number of follicles decreases (Fig. 4). This loss is thought to involve apoptosis, the rapidity of which makes detection of residual follicular debris difficult (Morita and Tilly, 1999). Ovaries from normal, *Zp1*-, *Zp2*- and *Zp3*-null mutant lines contained comparable numbers of primary and secondary stage follicles. However, all three null lines had statistically significant fewer late-stage follicles than normal (Fig. 4). In *Zp3*-null females, this difference was apparent at the early antral stage, and the two other null lines displayed a disparity at the mid-to-large antral stage of development. These data suggested that the disruption of the zona pellucida compromised the ability of follicles to continue development

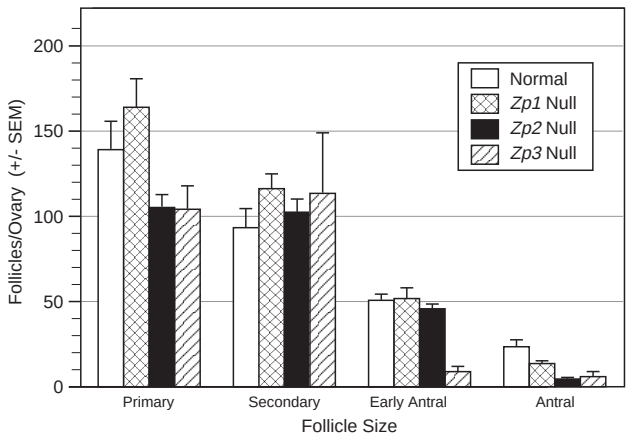

Fig. 4. Morphometric analysis of ovarian folliculogenesis. Single ovaries (four different animals) from normal, *Zp1*-null, *Zp2*-null and *Zp3*-null mice were serially sectioned and the number of primary, secondary, early antral and mid-to-late antral follicles was determined (Pedersen and Peters, 1968). Values are expressed as mean±s.e.m.

of antral follicles. Although absent (*Zp2*- or *Zp3*-null) or abnormal (*Zp1*-null) zonae pellucidae increased the fragility of oocytes under experimental conditions, it remained unclear whether the observed follicular loss was a direct effect of an abnormal or missing zona pellucida or an indirect effect, perhaps mediated by altered somatic-germ cell interactions.

In vitro fertilization and embryonic development in *Zp1*-, *Zp2*- and *Zp3*-null females

Because of the difficulty in collecting zona-free ovulated eggs from *Zp2*- and *Zp3*-null mice, oocytes were obtained by follicular puncture for all lines and matured in vitro. Even so, only five to six oocytes were isolated from each pair of *Zp2*- or *Zp3*-null ovaries, owing to reduced numbers of follicles and the extreme fragility of the zona-free oocytes. The in vitro matured eggs were fertilized and developed in vitro to the blastocyst stage, at which time they were transferred into the uteri of pseudopregnant females and allowed to continue to term (Table 2). No polyspermy was observed using a reduced concentration of sperm (5/μl) for eggs lacking a zona pellucida matrix due either to *Zp2*- or *Zp3*-null mutations or to biochemical removal from normal eggs.

Approximately 75% of eggs from *Zp1*-null mice were fertilized in vitro and developed to the two-cell stage, a rate that was comparable with that observed with normal eggs (81%). In contrast, only 50-53% of the zona-free eggs isolated from *Zp2*-null or the *Zp3*-null females progressed to the two-cell stage, a success rate similar to normal oocytes from which the zona pellucida had been removed by acidified Tyrode's medium (45%). A similar dichotomy, albeit less striking, was observed in progression from the two-cell stage to the blastocyst stage, with *Zp2*- and *Zp3*-null embryos less able to progress (30-41%) compared with *Zp1*-null embryos (50%). However, the zona-free blastocysts derived from *Zp2*- (or *Zp3*) null mice consistently appeared smaller and less healthy than those in which the zona pellucida was removed biochemically (Fig. 5). These observations suggest that oocyte development in the absence of ZP2 and ZP3 results in the production of eggs with reduced competence to undergo preimplantation development.

More striking was the absence of live births of pups derived from either *Zp2*- (31 transfers) or *Zp3*- (43 transfers) null eggs, although two (from *Zp3*-null eggs) were born dead (Table 2). In contrast, eggs from *Zp1*-null and normal eggs gave rise to live births after blastocyst transfers (14-27%), and even eggs that had been biochemically stripped of their zonae resulted in newborn mice (9%). Thus, it appears that the absence of ZP2 or ZP3 during folliculogenesis adversely affected the developmental competence of the *Zp2*- and *Zp3*-null eggs.

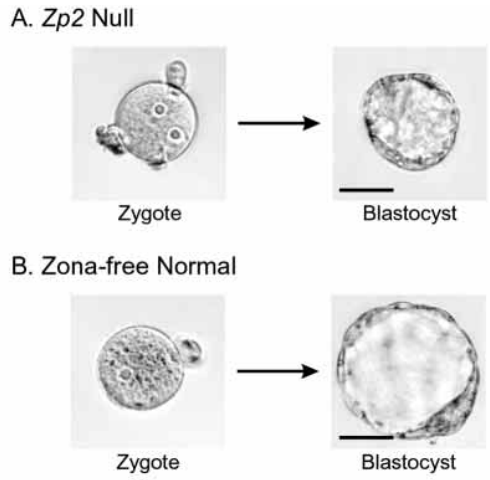

Fig. 5. Embryonic progression of *Zp2*-null eggs after fertilization. Oocytes were collected by follicular puncture of ovaries and matured in vitro. The zona pellucida was biochemically removed from a portion of the normal eggs (zona-free normal) and all were fertilized in vitro and cultured to the blastocyst stage. Photomicrographs of *Zp2*-null (A) and zona-free normal (B) zygotes with pronuclei and their progression to the blastocysts stage. Blastocysts derived from *Zp2*-null female mice were consistently smaller and less robust than those from normal mice. Scale bars: 50 μm.

DISCUSSION

Initially, *Zp2*-null mice develop a thin zona pellucida surrounding growing oocytes that is composed of ZP1 and ZP3. However, this matrix is not sustained through the antral stage of folliculogenesis and the ovulated eggs are zona-free. Although the early stages of folliculogenesis appear normal, the oophorus-cumulus complexes formed in preovulatory follicles are highly disorganized with loose, if any, association of the zona-free germ cell with the surrounding cumulus cells. Relatively few eggs (<5% of normal) are recovered in the oviduct after hormonal stimulation and the resultant sterility of *Zp2*-null females can be attributed to the paucity of eggs and their rapid absorption into the epithelial lining of the oviduct (Bronson and McLaren, 1970; Modlinski, 1970). Thus, the phenotype of the *Zp2*-null mice is distinct from that of *Zp1*-null mice (which form a zona matrix and are fertile) and that of *Zp3*-null mice (which never form a zona matrix and are sterile), although the severity of the defect is more similar to that of *Zp3*-null mice.

Despite intense investigation of the mouse zona pellucida since its biochemical composition was described (Bleil and

Table 2. Progression of embryogenesis in *Zp1*-, *Zp2*- and *Zp3*-null mice

	Normal	*Zp1* null	*Zp2* Null	*Zp3* null	Zona-free normal
Fertilized eggs	518 (100%)	452 (100%)	227 (100%)	395 (100%)	395 (100%)
Two-cell embryos*	417 (81%)	337 (75%)	120 (53%)	196 (50%)	179 (45%)
Blastocysts*	253 (61%)	170 (50%)	36 (30%)	81 (41%)	72 (40%)
Transferred‡	164 (100%)	162 (100%)	31 (100%)	43 (100%)	56 (100%)
Live births§	24 (15%)	44 (27%)	0 (0%)	0 (0%)	5 (9%)

*Number of embryos (percent that progress from previous stage).
‡Number of cultured blastocysts transferred to pseudopregnant females.
§Number of live births (percent of transferred blastocysts).

Wassarman, 1980; Shimizu et al., 1983), little is known about its formation or three-dimensional structure (for review, see Green, 1997). ZP1 (623 amino acids), ZP2 (713 amino acids) and ZP3 (424 amino acids) differ from one another, but share specific defining motifs (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 260 amino acid 'zona box' (Bork and Sander, 1992) and each has a transmembrane domain near its C terminus, 20-30 amino acids upstream of which is a potential endoproteolytic cleavage site (Yurewicz et al., 1993). In addition, the 348 amino acid C termini of ZP1 (residues 268-623) and ZP2 (residues 363-713) are 32% identical (47% similar) suggesting that they arose from a common ancestral gene (Epifano et al., 1995a). Although the stoichiometry of the three zona pellucida proteins within the matrix has not been accurately determined, it appears that there is significantly less ZP1 than either ZP2 or ZP3 (Green, 1997).

Mice that lack ZP2 can form a zona matrix composed of ZP1 and ZP3 early in oogenesis, but it is considerably thinner and does not persist in ovulated eggs, unlike the matrix formed by ZP2 and ZP3 in *Zp1*-null mice (Rankin et al., 1999). Mice without ZP3, however, do not form a zona matrix, even early in oogenesis (Liu et al., 1996; Rankin et al., 1996). These data suggest that two zona pellucida proteins are sufficient for zona matrix formation. One must be ZP3, but the other can be either ZP1 or ZP2. The thinness of the ZP1/ZP3 matrix compared with the ZP2/ZP3 matrix could result either from a more enduring interaction between ZP2 and ZP3, or limiting amounts of ZP1. The latter hypothesis is consistent with there being 20-25% as many transcripts encoding ZP1 as there are encoding ZP2 or ZP3 in growing oocytes (Epifano et al., 1995b). If the abundance of ZP1 protein limits zona pellucida thickness, it may be possible to augment the zona matrix of the *Zp2*-null mice and reverse the sterile phenotype by overexpression of ZP1 in transgenic mice.

Unexpectedly, zona-free oocytes from both *Zp2*- or *Zp3*-null mice appear to be developmentally compromised, suggesting that the absence of a zona pellucida has an adverse maternal affect on embryogenesis that arises during folliculogenesis. Morphometric analysis of ovarian follicles indicates that oocytes with structurally defective (*Zp1*- or *Zp2*-null) or completely absent (*Zp3*-null) zona matrices progress normally through the pre-antral stages of folliculogenesis, although by the antral stages, there is a significant decrease in the number of surviving follicles. Growing oocytes isolated from each of the null lines can be matured and fertilized in vitro and some will progress to the blastocyst stage in culture (albeit the blastocyst quality is poor). After transfer to foster mothers, embryos encased in structurally abnormal zonae (*Zp1* null) result in live births, but those lacking a zona pellucida (*Zp2* or *Zp3* null) do not complete development. The birth of pups derived from control oocytes from which the zona pellucida had been removed biochemically (this manuscript, Naito et al., 1992) suggests that this developmental defect(s) is not attributable only to the physical absence of a zona pellucida matrix during embryogenesis.

During folliculogenesis, surrounding granulosa cells interact with oocytes via processes that penetrate through the zona pellucida matrix and form gap junctions with the plasma membrane (Anderson and Albertini, 1976; Eppig, 1991). These interactions provide two-way communications required for oocyte growth, and maintain the oocyte in meiotic arrest (oocytes released from late stage follicles will spontaneously undergo the completion of the first meiotic division). Oocytes unable to form interactions with surrounding granulosa cells, either because of ectopic displacement (Zamboni and Upadhyay, 1983) or genetic mutation (Soyal et al., 2000), do not survive. While there is long-standing evidence that granulosa cells support oocyte growth and maturation (reviewed by Eppig et al., 1997a), the reciprocal phenomenon of oocytes modulating granulosa and cumulus cell functions has become clear only over the past decade.

In mutant mice that lack germ cells, follicles do not form (Columbre and Russell, 1954) and pharmacological ablation of oocytes in rats results in defective folliculogenesis (Merchant, 1975; Hirshfield, 1994). Factor(s) secreted by oocytes promote granulosa cell proliferation and differentiation (Vanderhyden et al., 1990; Vanderhyden et al., 1992; Eppig et al., 1997a). One, GDF9, an oocyte-specific member of the transforming growth factor β family, is required for the proliferation of granulosa cells beyond the primary stage of folliculogenesis and for the recruitment of thecal cells to the follicle (Dong et al., 1996; Elvin et al., 1999). Oocyte factors also promote preovulatory cumulus mucification by enabling cumulus cells to secrete hyaluronic acid in response to follicle-stimulating hormone (Salustri et al., 1990; Buccione et al., 1990). Additionally, oocytes have been implicated in modulating specific gene expression (i.e. that of the genes for the luteinizing hormone receptor and Kit ligand) in surrounding granulosa cells (Eppig et al., 1997b; Joyce et al., 1999). Taken together, these data strongly support the hypothesis that oocytes and follicular somatic cells are interdependent throughout their development.

Although the fragility of the zona-free null oocytes precluded assessment of metabolic coupling between somatic and germ cells, it seems likely that both physical and metabolic perturbations affect the developmental potential of the egg. A relatively high percent (75-81%) of fertilized eggs with a normal or even an abnormal zona pellucida (*Zp1* null) progress to the two cell stage. Far fewer eggs without a zona pellucida (45-53%) progress to two-cell embryos, whether the absence of a zona pellucida results from a genetic perturbation (*Zp2* or *Zp3* null) or biochemical removal (zona-free normal). These observations are consistent with the physical absence of the zona pellucida compromising very early embryonic progression. Although zona-free two-cell embryos progress to the blastocyst stage in vitro, no live births resulted from the transfers of *Zp2*- and *Zp3*-null blastocysts to foster mothers despite the observation that normal zona-free blastocysts result in live pups, albeit at a rate somewhat less than zona-intact embryos (this manuscript, Naito et al., 1992). These data suggest that it is the absence of a zona pellucida matrix during folliculogenesis that adversely affects the developmental potential of the *Zp2*- and *Zp3*-null embryos after transfer to foster mothers. The genetic pathways by which such a perturbation could occur, remain to be determined.

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