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

Tracy L. Rankin1,*, Marilyn O’Brien2, Eric Lee3, Karen Wigglesworth2, John Eppig2 and Jurrien Dean1

1Laboratory of Cellular and Developmental Biology, NIDDK, National Institutes of Health, Bethesda, MD 20892, USA
2The Jackson Laboratory, Bar Harbor, ME 04607, USA
3Laboratory of Mammalian Genes and Development, NICHD, National Institutes of Health, Bethesda, MD 20892, USA
*Author for correspondence (e-mail: tr44x@nih.gov)

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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, arrest 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 stoichiometric 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 Pmel-Kpm1 fragment containing a portion of the Zp2 promoter and exon 1 (including the ATG initiation site) was subcloned into the XhoI site of pPNT and a second, 5 kb EcoRI-SpeI fragment containing exons 3-7, was cloned into the XbaI site (Fig. 1). The targeting construct was linearized with NorI and electroporated into R1 embryonic stem cells (Nagy et al., 1993). Individual clones were selected after growth in G418 (Life Technologies, Rockville, MD) and gancyclovir (Roche Discovery, Welwyn, UK), and correctly targeted clones were identified by Southern blot analysis with 32P-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 PstI fragment isolated from PGK-Neo. Heterozygous Zp2<sup>tm/+</sup> cells from multiple lines were injected into C57BL/6N blastocysts to obtain coat color lines were injected into C57BL/6N blastocysts to obtain coat color lines were injected into C57BL/6N blastocysts to obtain coat color lines were injected into C57BL/6N blastocysts to obtain coat color lines were injected into C57BL/6N blastocysts to obtain coat color lines were injected into C57BL/6N blastocysts to obtain coat color.

Ovarian histology

Ovaries 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<sub>1</sub> 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<sub>2</sub>HPO<sub>4</sub>), 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, 4°C) 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 Zeiss 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
Developmental competence of Zp2-null eggs 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 selection with G418 and gancyclovir (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 F1 heterozygotes. Male and female heterozygotes were crossed and produced Zp2+/-, Zp2+/tm and Zp2tm/tm offspring in the expected Mendelian ratios for transmission 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 (Zp2tm1Nih/tm1Nih and Zp2tm2Nih/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...
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

<table>
<thead>
<tr>
<th></th>
<th>Ovulated eggs*</th>
<th>Two-cell embryos*</th>
<th>Live births‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (Zp2+/+)</td>
<td>35.6±7.7 (8/8)</td>
<td>17.6±6.1 (8)</td>
<td>8.6±0.6 (31)</td>
</tr>
<tr>
<td>Homozygous null (Zp2tm/tm)</td>
<td>1.4±0.6 (6/12)</td>
<td>0 (7)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*Average±s.e.m. (number of animals from which eggs recovered/number of animals).
‡Average±s.e.m. (number of litters).
Developmental competence of Zp2-null eggs

1123

Developmental competence of Zp2-null eggs

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 stoichiometric 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.
of antral follicles. Although absent (Zp2- or Zp3-null) or abnormal (Zp1-null) zona pellucida 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 uterus of pseudopregnant females and allowed to continue to term (Table 2). No polyspermy was used reducing a 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 zona 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.

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

<table>
<thead>
<tr>
<th>Stage</th>
<th>Normal</th>
<th>Zp1 null</th>
<th>Zp2 null</th>
<th>Zp3 null</th>
<th>Zona-free normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fertilized eggs</td>
<td>518 (100%)</td>
<td>452 (100%)</td>
<td>227 (100%)</td>
<td>305 (100%)</td>
<td>395 (100%)</td>
</tr>
<tr>
<td>Two-cell embryos*</td>
<td>417 (81%)</td>
<td>337 (75%)</td>
<td>120 (53%)</td>
<td>196 (50%)</td>
<td>179 (45%)</td>
</tr>
<tr>
<td>Blastocysts*</td>
<td>253 (61%)</td>
<td>170 (50%)</td>
<td>36 (30%)</td>
<td>81 (41%)</td>
<td>72 (40%)</td>
</tr>
<tr>
<td>Transferred‡</td>
<td>164 (100%)</td>
<td>162 (100%)</td>
<td>31 (100%)</td>
<td>43 (100%)</td>
<td>56 (100%)</td>
</tr>
<tr>
<td>Live births§</td>
<td>24 (15%)</td>
<td>44 (27%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>5 (9%)</td>
</tr>
</tbody>
</table>

*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 thickness 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.

REFERENCES


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