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Targeted disruption of the *mZP3* gene results in production of eggs lacking a zona pellucida and infertility in female mice

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ABSTRACT **Mammalian eggs are surrounded by a thick extracellular coat, the zona pellucida, that plays important roles during early development. The mouse egg zona pellucida is constructed of three glycoproteins, called mZP1, mZP2, and mZP3. The gene encoding mZP3 is expressed only by growing oocytes during a 2- to 3-week period of oogenesis. Here, the *mZP3* gene was disrupted by targeted mutagenesis using homologous recombination in mouse embryonic stem cells. Viable female mice homozygous for the mutated mZP3 allele (*mZP3*$^{-/-}$) were obtained. These mice are indistinguishable in appearance from wild-type (*mZP3*$^{+/+}$) and heterozygous (*mZP3*$^{+/-}$) littermates. However, although ovaries of juvenile and adult *mZP3*$^{-/-}$ females possess growing and fully grown oocytes, the oocytes completely lack a zona pellucida. Consistent with this observation, eggs recovered from oviducts of superovulated, adult *mZP3*$^{-/-}$ females also lack a zona pellucida. Thus far, *mZP3*$^{-/-}$ females mated with wild-type males have failed to become pregnant.**

The zona pellucida (ZP) is a relatively thick extracellular coat that surrounds the plasma membrane of mammalian eggs (1-4). The ZP plays important roles during fertilization, oogenesis, and preimplantation development. For example, the ZP serves as a barrier to fertilization of eggs by sperm from a different species (heterospecific fertilization) and by more than one sperm from the same species (polyspermic fertilization). Apparently, the ZP serves a protective function as the embryo makes its way toward the uterus during the preimplantation stages of development (5, 6).

The ZP is laid down during the final stages of oogenesis when nongrowing oocytes enter their 2- to 3-week growth phase (7). As oocytes increase in diameter, from ≈15 to 80 μm in diameter, the ZP increases in thickness. The mouse egg ZP is ≈7 μm thick, contains about 3 ng of protein, and consists of an extensive network of interconnected filaments. It is constructed of three glycoproteins, called mZP1, mZP2, and mZP3, that are synthesized and secreted by growing mouse oocytes (8, 9). Two of the glycoproteins, mZP2 and mZP3, form long filaments and the third, mZP1, connects the filaments (10). Interactions between ZP glycoproteins are noncovalent in nature.

Free-swimming, acrosome-intact mouse sperm recognize and bind to mZP3 during fertilization (11-13). Sperm bind to a specific class of Ser/Thr-(O)-linked oligosaccharides located on the carboxyl-terminal third of the mZP3 polypeptide (14, 15). Once bound to mZP3, sperm are induced to undergo the acrosome reaction; a form of cellular exocytosis that enables sperm to penetrate the ZP and reach and fuse with the plasma membrane (12, 16-18).

The gene encoding mZP3 has been cloned and characterized and some of its regulatory elements responsible for oocyte-specific expression have been identified (19, 20). Here, we describe the introduction of a null mutation in the *mZP3* gene by homologous recombination in embryonic stem (ES) cells. The phenotype of female mice homozygous for the null mutation (*mZP3*$^{-/-}$) includes the complete absence of a ZP on growing oocytes. This finding is consistent with the structural role proposed for mZP3 during ZP assembly (10, 21). A preliminary report of these results has appeared (22).

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MATERIALS AND METHODS

Vector Construction for Targeted Mutagenesis. The *mZP3* gene from 129/Sv mice was cloned by screening a lambda phage genomic DNA library with a ^{32}P-labeled cDNA probe prepared using the CD-1 mouse *mZP3* gene. The 129/Sv mouse *mZP3* gene then was used as template to prepare 5′- and 3′-fragments, 2.6 kb and 4.3 kb, respectively, by polymerase chain reaction (PCR) amplification. The 2.6 kb 5′-fragment includes 289 bp of exon 1 (out of 327 bp), 85 bp of exon 2 (out of 119 bp), and all of intron 1 (≈2.2 kb) (see Fig. 1). The 4.3 kb 3′-fragment includes 27 bp of exon 3 (out of 104 bp), 200 bp of exon 8 (out of 219 bp), and all of exons 4-7 and introns 3-7 (see Fig. 1). The 5′- and 3′-fragments were ligated to the upstream and downstream regions, respectively, of the pGKneobpa expression cassette (23). This cassette serves as a positive selection marker because it enables ES cells that contain the targeting vector to grow in the presence of G418. To select against random integration of the targeting vector, pMCI-HSV-TK cassette, a negative selection marker, was ligated downstream of the 3′-fragment (24). In cases of random integration of the targeting vector, expression of the herpes simplex virus thymidine kinase gene prevents growth of ES cells in medium containing 1-[2′-deoxy-2′-fluoro-β-D-arabinofuranosyl]-5′-iodouracil (FIAU). The targeting vector was linearized with *Cla*I before electroporation into ES cells.

Transfection, Selection, and Screening of ES Cells. Mouse ES cells (W.9.5; ref. 25) were cultured with feeder cells (primary mouse embryonic fibroblasts) in M15 medium (Dulbecco's modified Eagle's medium with high glucose and glutamine, and without sodium pyruvate and with 15% fetal bovine serum/0.1 mM β-mercaptoethanol/50 units per ml penicillin/50 units per ml streptomycin) under standard con-

Abbreviations: ZP, zona pellucida; ES cells, embryonic stem cells.

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ditions (26). Approximately 3×10^7 ES cells in log-phase of growth were harvested and transfected by electroporation (Bio-Rad model Gene Pulser; 250 μF, 320 V/cm) with 30 μg of linearized DNA targeting vector. Electroporated ES cells were plated onto γ-ray irradiated primary mouse embryonic fibroblasts and cultured in M15 medium containing G418 (350 μg/ml; neomycin-positive selection) and FIAU (2 μM; thymidine kinase-negative selection). After 7 days of selection, 96 ES cell colonies were individually picked and expanded in 48-well microtiter dishes for another 5 days. At confluency, each ES cell clone was trypsinized and one-fifth of the cells were stored in liquid nitrogen; the remainder of the cells was harvested and incubated overnight at 56°C in lysis buffer (10 mM Tris·HCl, pH 8.3/50 mM KCl/2.5 mM $MgCl_2$/0.45% Tween-20/0.45% Nonidet P-40/60 μg/ml proteinase K).

Genomic DNA was isolated from ES cell lysates by phenol/chloroform extraction and ethanol precipitation. To screen targeted ES cell lines, DNA was amplified by PCR using a Gene Amp PCR kit (Perkin–Elmer/Cetus) and Taq extender (Stratagene). As depicted in Fig. 1, the upstream PCR primer used (CTGAGCCCAGCTGTACTCCAGGCG) can hybridize to a portion of *mZP3* gene exon 1 just upstream of the 5′-targeting fragment. The downstream PCR primer used (GCTACCGGTGGATGTGGAATGTGTGC) can hybridize to the PGK promoter region of the pGKneobpa marker within the targeting vector. PCR was run for 35 cycles of 25 sec at 95°C, 30 sec at 63°C, and 3 min at 72°C, and the products were analyzed by 1% agarose gel electrophoresis. Amplification of a 2.7-kb DNA fragment is indicative of homologous recombination. PCR positive clones were confirmed by Southern blot analyses essentially as described (28). Briefly, ES cell genomic DNA was digested with *Eco*RI, subjected to electrophoresis on 0.8% agarose gels, and transferred onto nitrocellulose membrane. The membrane was baked and prehybridized, incubated in the presence of a ^{32}P-labeled mZP3 DNA probe (Megaprime DNA Labeling System; Amersham) that hybridizes to the first 271 bp of mZP3 gene exon 1, washed, and exposed to x-ray film. The presence of a 2.7-kb band due to the introduction of an *Eco*RI site (Fig. 1), in addition to a 10-kb wild-type band, indicates that the *mZP3* gene was disrupted correctly.

Production of Mice Homozygous for the Null Mutation. Six positive ES cell clones were thawed and expanded. ES cells in log-phase of growth were trypsinized and resuspended in blastocyst injection buffer (similar to M15, except that 25 mM Hepes and 10% fetal bovine serum were present). ES cells (10-20) were injected into the blastocoel cavity of each 3.5-day

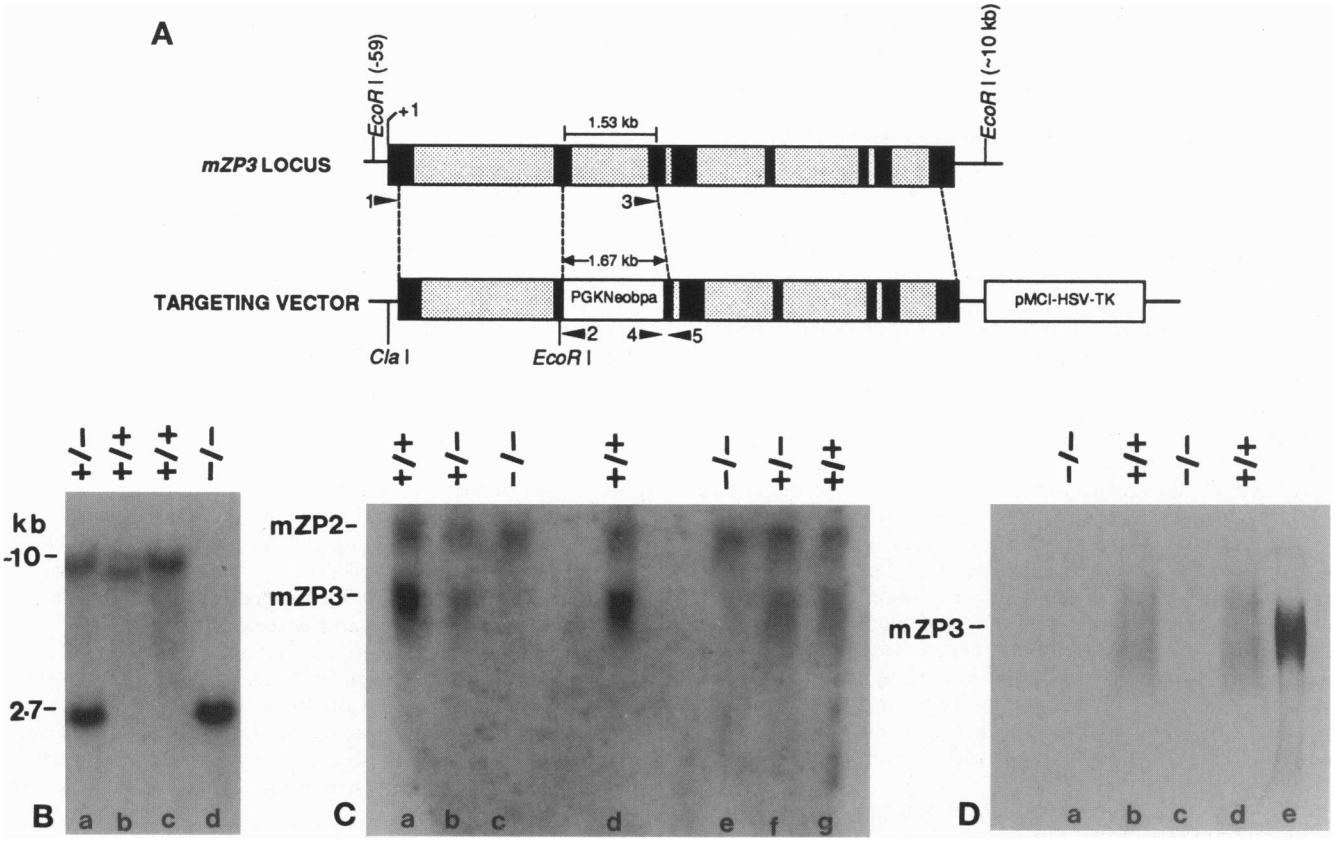

FIG. 1. Targeted disruption of the *mZP3* gene and analysis of offspring. (*A*) Schematic representation of the *mZP3* locus and the targeting vector. Black boxes represent exons, stippled boxes represent introns, and lines represent upstream and downstream flanking sequences of the *mZP3* gene. In the targeting vector, portions of exon 2 and exon 3, and the entire intron 2, were replaced by the pGKneobpa expression cassette in order to disrupt the *mZP3* gene. The location of the negative selection marker, pMCI-HSV-TK cassette, and the restriction sites for *Eco*RI and *Cla*I are also shown. The numbered arrows indicate the relative positions of oligonucleotide primers used in PCR. Primers 1 and 2 were used to screen targeted ES cell clones and primers 3-5 were used to screen heterozygous ($mZP3^{+/-}$) and homozygous ($mZP3^{-/-}$) mutant mice. (*B*) Southern blot analysis of mouse genomic DNA. Purified mouse genomic DNA was digested with *Eco*RI and then incubated with a ^{32}P-labeled mZP3 DNA fragment that can hybridize to the first 271 bp of mZP3 exon 1. The designations +/+, +/−, and −/− indicate wild-type, heterozygous, and homozygous mutant mice, respectively. Note the presence of a single 2.7-kb band in $mZP3^{-/-}$ mice (lane d). (*C*) Northern blot analysis of mouse ovarian RNA. Ovarian RNA samples were incubated with ^{32}P-labeled probes directed against mZP2 and mZP3 mRNAs (27). The designations +/+, +/−, and −/− indicate wild-type, heterozygous, and homozygous mutant mice, respectively. Note the absence of mZP3 mRNA only in $mZP3^{-/-}$ mice (lanes c and e). (*D*) Western blot analysis of ovarian mZP3 using a polyclonal goat antibody directed against the glycoprotein (27). The designations +/+ and −/− indicate wild-type and homozygous mutant mice, respectively. Note the absence of mZP3 in $mZP3^{-/-}$ mice (lanes a and c). Lane e contains ≈100 μg HPLC-purified mZP3 (≈83,000 M_r). There is ≈10-times more mZP3 in lane e than in lanes b and d.

blastocyst collected from C57BL/6J mice (black). Injected blastocysts were transferred into uteri of 2.5 day pseudopregnant B6CBAF1/J foster mothers. Male chimeras with significant agouti coat color born to foster mothers were mated with C57BL/6J females (black) and germ-line transmitted progeny (agouti) were analyzed by PCR using tail DNA to identify heterozyous mutant mice ($mZP3^{+/-}$). An upstream primer corresponding to the bpa region of the pGKneobpa marker within the targeting vector (GGAAGACAATAGCAGGCATGCTGGG) and a downstream primer corresponding to mZP3 gene exon 4 (CCCGTGGAAGTCCACGATGAAGTGAT) were used (Fig. 1). PCR was run for 35 cycles of 30 sec at 94°C, 30 sec at 59°C, and 45 sec at 72°C. DNA from heterozygous mutant mice yields a 410-bp band. To produce homozygous mutant mice ($mZP3^{-/-}$), male and female heterozygous mutant mice were mated and their offspring analyzed by PCR using tail DNA. PCR conditions were similar to those used to analyze heterozygous mice, as described above, except that an additional upstream primer corresponding to *mZP3* gene exon 3 (GCCACTGTGTCCTCAGAGGAG) was added. Because this region of mZP3 is not part of the targeting vector (Fig. 1), this primer will hybridize only to wild-type mouse DNA and result in amplification of a 319-bp band. Therefore, by using these three PCR primers, DNA from homozygous ($mZP3^{-/-}$), wild-type ($mZP3^{+/+}$), and heterozygous ($mZP3^{+/-}$) mice results in amplification of a 410-bp band, a 319-bp band, and 319-bp and 410-bp bands, respectively. In some cases, Southern blot analysis was used in conjuction with the PCR screens.

Southern, Northern, and Western Blot Analyses. Southern blot analysis was carried out as described (28). Northern blot analysis was carried out with ovarian RNA, separated by electrophoresis on 1.5% agarose/2.2 M formaldehyde gels, transferred to nitrocellulose, and probed with ^{32}P-labeled, full-length cDNAs, as described (27). Western blot analysis was carried out with ovarian homogenates subjected to SDS/PAGE, transferred to nitrocellulose, and probed with a polyclonal goat anti-mZP3 IgG followed by a rabbit anti-goat IgG alkaline phosphatase conjugate (Bio-Rad) and NBT/BCIP (GIBCO/BRL) as described (27).

Light and Electron Microscopic Analyses. Ovaries were excised from mice and placed in 4% paraformaldehyde/20 mM ethylacetimidate for 2 hr followed by 2% glutaraldehyde in PBS for an additional 2 hr. The ovaries were then washed, placed in 1% OsO_4 in PBS for 2 hr, washed, and passed through a graded ethanol series from 30% to 100% ethanol. They were then immersed in propylene oxide for two 15-min periods and then in propylene oxide/epon at 1:1 for 30 min and propylene oxide/epon at 1:2 for 30 min. Ovaries were immersed in epon for 2 hr, placed in beem capsules, and polymerized overnight at 65°C. Thick sections (≈0.5 μm) were cut with glass knives and counterstained with 1% toluidine blue in borate buffer. Thin sections were cut with diamond knives, placed on copper grids, and counterstained with 4% uranyl acetate in water. Thick sections were examined with a Zeiss Universal microscope and thin sections with a JEOL 1200EX electron microscope.

RESULTS

Experimental Rationale. At about the time of birth, mouse ovaries are populated by a large pool of nongrowing oocytes (≈15 μm in diameter) arrested in the first meiotic prophase and lacking a ZP (7). During the first 21 days or so after the birth of female mice ("juvenile animals"), an unusually large number of oocytes begin to grow and, concomitantly, to lay down a ZP. The diameter of growing oocytes isolated from ovaries is directly related to the age of the donor mice and the thickness of the ZP is directly related to oocyte diameter during days 3 to 21 postpartum (7, 29).

The three mouse ZP glycoproteins, mZP1, mZP2, and mZP3, are synthesized and secreted by oocytes throughout their growth phase (30-33). In fact, the *mZP3* gene is expressed at very high levels exclusively in growing oocytes, with mZP3 mRNA reaching a steady-state level of nearly 250,000 copies in each fully grown oocyte (≈80 μm in diameter) (34). Here, we disrupted the *mZP3* gene by targeted mutagenesis using homologous recombination in ES cells to determine, among other things, whether mZP3 is essential for construction of a ZP during oocyte growth. Female mice homozygous for the mutated mZP3 allele ($mZP3^{-/-}$) were obtained, and growing oocytes of both juvenile (3 to 21 days old) and adult animals (>4 weeks old) were examined.

Generation and Analysis of Female $mZP3^{-/-}$ Mice. mZP3 null mutant mice ($mZP3^{-/-}$) were produced using homologous recombination in ES cells by following standard gene targeting procedures (26, 35) (see *Materials and Methods*). The targeting vector was constructed such that part of mZP3 exon 2 and exon 3, and all of mZP3 intron 2, were replaced by the positive selection marker pGKneobpa cassette (Fig. 1*A*). A total of 96 ES cell colonies were screened by PCR and then confirmed by Southern blot analysis. Six positive ES cell lines were injected into mouse blastocysts and all were transmitted through the germ line. Heterozygous mutant mice ($mZP3^{+/-}$) were identified by PCR using tail-DNA and homozygous female mutant mice ($mZP3^{-/-}$) were identified in a similar manner, screening pups born to a pair of heterozygous mice. In several instances, PCR results were confirmed by Southern blot analysis (e.g., Fig. 1*B*). $mZP3^{-/-}$ female mice were analyzed by Northern blotting (Fig. 1*C*) and Western blotting (Fig. 1*D*) to establish that mZP3 mRNA (≈1.5 kb) and glycoprotein (≈83,000 M_r), respectively, were not present in growing oocytes. As seen in Fig. 1*C*, whereas mZP3 mRNA was present in ovaries taken from wild-type and heterozygous mutant mice, it was not found in ovaries taken from homozygous mutant mice. Similarly, mZP3 glycoprotein was undetectable in ovaries excised from homozygous mutant mice (Fig. 1*D*). It should be noted that $mZP3^{-/-}$ mice were indistinguishable in appearance from wild-type and heterozygous littermates, and exhibited normal growth and development.

Growing Oocytes of Juvenile Female $mZP3^{-/-}$ Mice. Ovaries were excised from female $mZP3^{-/-}$ mice 14 to 21 days old (juvenile mice) and growing oocytes were examined by light microscopy. Certain phenotypic changes were noted for ovaries and oocytes removed from null mutant, juvenile mice. Ovaries excised from 14- to 20-day-old $mZP3^{-/-}$ mice were about one-third to one-half the size of ovaries from wild-type and heterozygous mice of the same age (data not shown). When growing oocytes were isolated from ovaries of 17-day-old $mZP3^{-/-}$ mice, by poking ovaries with fine steel needles, they were found to be about the same size as oocytes from ovaries of wild-type mice (≈68 μm in diameter); however, the oocytes did not have a ZP (Figs. 2*A* and 3). The absence of a ZP was characteristic of oocytes isolated from all juvenile $mZP3^{-/-}$ mice.

Fully Grown Oocytes, Eggs, and Follicles of Adult Female $mZP3^{-/-}$ Mice. Ovaries were excised from 4- to 6-week-old female $mZP3^{-/-}$ mice (adult mice) and oocytes were obtained by poking ovaries with fine steel needles and examined by light microscopy. These ovaries were about the same size as ovaries from wild-type and heterozygous mice of the same age (data not shown). On the other hand, although oocytes isolated from ovaries of 4- to 6-week-old $mZP3^{-/-}$ mice were about the same size as oocytes from ovaries of wild-type mice, the oocytes did not have a ZP (see below). These observations strongly suggest that, while oocytes of $mZP3^{-/-}$ mice grow to full-size (≈80 μm in diameter), they do not produce a ZP. Consistent with this conclusion, while very few eggs were recovered from oviducts of superovulated $mZP3^{-/-}$ mice (e.g., ≈2 eggs per $mZP3^{-/-}$ mouse versus ≈20 eggs per superovulated $mZP3^{+/-}$ or

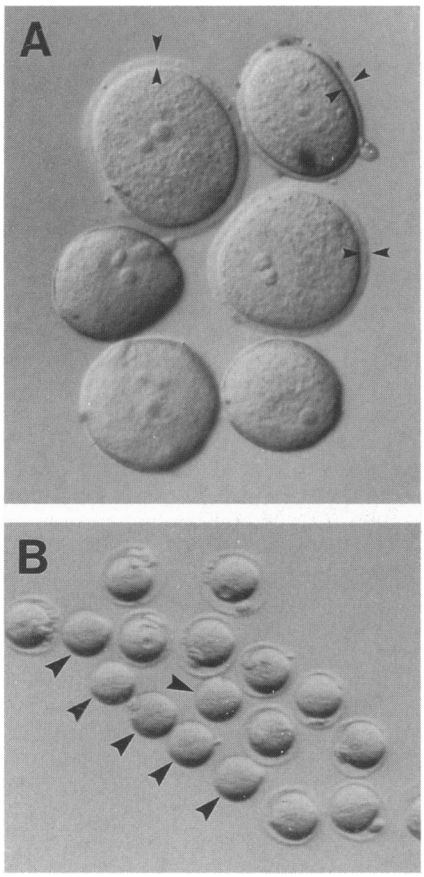

FIG. 2. Light micrographs of growing oocytes and unfertilized eggs recovered from *mZP3*$^{-/-}$ and wild-type mice (*A*) Growing oocytes were obtained by poking ovaries excised from 17-day-old (juvenile) female mice and were then fixed in 1% paraformaldehyde. Shown are three oocytes with a ZP from wild-type mice (arrowheads indicating the ZP) and three oocytes lacking a ZP from *mZP3*$^{-/-}$ mice. (*B*) Unfertilized eggs were obtained from oviducts excised from ≈6 week-old, superovulated female mice. Shown are six ovulated eggs recovered from *mZP3*$^{-/-}$ mice (arrowheads; lacking a ZP) and 12 ovulated eggs recovered from wild-type mice. Micrographs were taken using Nomarski differential interference contrast microscopy. (*A*, × ≈260; *B*, ×≈85.)

mZP3$^{+/+}$ mouse), any eggs that were retrieved from cumulus masses in oviducts of *mZP3*$^{-/-}$ mice also lacked a ZP (Fig. 2*B*).

Both light and electron microscopy were used to examine follicle development in female *mZP3*$^{-/-}$ mice. As seen in Fig. 3, follicle development in *mZP3*$^{-/-}$ mice (Fig. 3*D*) was very similar to follicle development in wild-type mice (Fig. 3*C*). In each case, the fully grown oocyte was contained within a multilayered, antral follicle. Aside from the absence of a ZP on *mZP3*$^{-/-}$ oocytes, the most obvious difference between follicles in *mZP3*$^{-/-}$ and wild-type mice was the degree of contact between the oocyte and follicle cells. In the latter case, the oocyte was completely surrounded by follicle cells that were in very close contact with the oocyte. In *mZP3*$^{-/-}$ mice, the portion of the oocyte facing the follicular antrum was naked and the degree of contact between the rest of the oocyte and follicle cells was diminished. The latter is seen more clearly in the electron micrographs shown in Fig. 3. In wild-type mice (Fig. 3*E*), follicle cells are in very close apposition to the oocyte's ZP, sending projections through the ZP to connect with the oocyte. In *mZP3*$^{-/-}$ mice (Fig. 3*F*), the oocyte does not appear to be intimately associated with follicle cells. The follicle cells send out a few projections and, although the oocyte has numerous microvilli, there appears to be a distinct region separating the oocyte from follicle cells.

Reproduction of Female *mZP3*$^{-/-}$ Mice. To determine whether or not female *mZP3*$^{-/-}$ mice are fertile, homozygous null mutant animals were placed with wild-type males. Fourteen *mZP3*$^{-/-}$ females were kept with fertile males for more than 3 months and, although copulation plugs were frequently observed, none of the females became pregnant during this period. Therefore, for reasons that are not altogether clear (see *Discussion*), the *mZP3*$^{-/-}$ females are infertile.

DISCUSSION

Growing oocytes do not produce a ZP in mice carrying a homozygous null mutation for the *mZP3* gene. Despite this, for female *mZP3*$^{-/-}$ mice, oocytes grow to about full-size (≈80 μm in diameter), follicles develop to the antral stage, and mice ovulate in response to gonadotropin injection. Therefore, construction of a ZP is not essential for either oocyte growth or follicle development. The former point is consistent with the observation that oocytes located at an ectopic site, not contained within an ovarian follicle, undergo growth (36). On the other hand, female *mZP3*$^{-/-}$ mice are infertile and the infertility appears to be related to the absence of a ZP on ovulated eggs. Examination of superovulated *mZP3*$^{-/-}$ females revealed that, while a cumulus mass was always found in their oviducts, frequently no eggs or only a few eggs were present within the cumulus mass. This finding is consistent with the observation that *mZP3*$^{-/-}$ oocytes are not intimately associated with surrounding follicle cells in the ovary. It is likely that eggs lacking a ZP are lost during ovulation, either by sticking to the reproductive tract or by atresia. Those eggs that succeed in making it to the oviduct are either not fertilized or are fertilized, but fail to progress normally through early pregnancy. However, a much more detailed analysis of this feature of female *mZP3*$^{-/-}$ mice is needed in order to account unambiguously for their infertility.

The ZP is laid down during oocyte growth and all three mouse ZP glycoproteins are synthesized and secreted by growing oocytes, not by follicle cells. Previously, we reported that the mouse egg ZP consists of long filaments, composed of mZP2 and mZP3, that are interconnected by mZP1 (10, 21). The filaments exhibit a structural periodicity in electron micrographs that apparently reflects the presence of an mZP2-mZP3 heterodimer every 15 nm or so along the filaments. Therefore, mZP3 is an abundant (>10^9 copies per ZP) structural component of the ZP. Consequently, it is not surprising that disruption of the mZP3 gene results in production of oocytes lacking a ZP. In this context, a recent report suggests that inhibition of either mZP2 or mZP3 synthesis, using antisense oligonucleotides complementary to the 5′-ends of mZP2 and mZP3 mRNA, prevented incorporation of the other glycoprotein into the oocyte ZP (37). It can be concluded from results obtained here with *mZP3*$^{-/-}$ mice, as well as from these antisense experiments, that mZP2 and mZP3 must be synthesized concomitantly in order to assemble the filaments that constitute the ZP. It is of interest to note that, despite the complete absence of mZP3 synthesis, mZP2 is synthesized by growing oocytes of female *mZP3*$^{-/-}$ mice (data not shown).

Each nongrowing mouse oocyte is contained within a cellular follicle that grows concomitantly with the oocyte, from a single layer of a few epithelial-like cells to several layers of cuboidal granulosa cells (~900 cells) by the time the oocyte has completed its growth (6). During a period of several days, while the oocyte remains a constant size, the number of follicle cells increases to more than 50,000 and results in a follicle about 600 μm in diameter. The follicle exhibits an incipient antrum when it is several layers thick (~6,000 cells) and, as the antrum expands, the oocyte takes up an acentric position surrounded by two or more layers of cells. The innermost layer of cells extends processes through the ZP and forms specialized gap junctions with the oocyte plasma membrane. Since

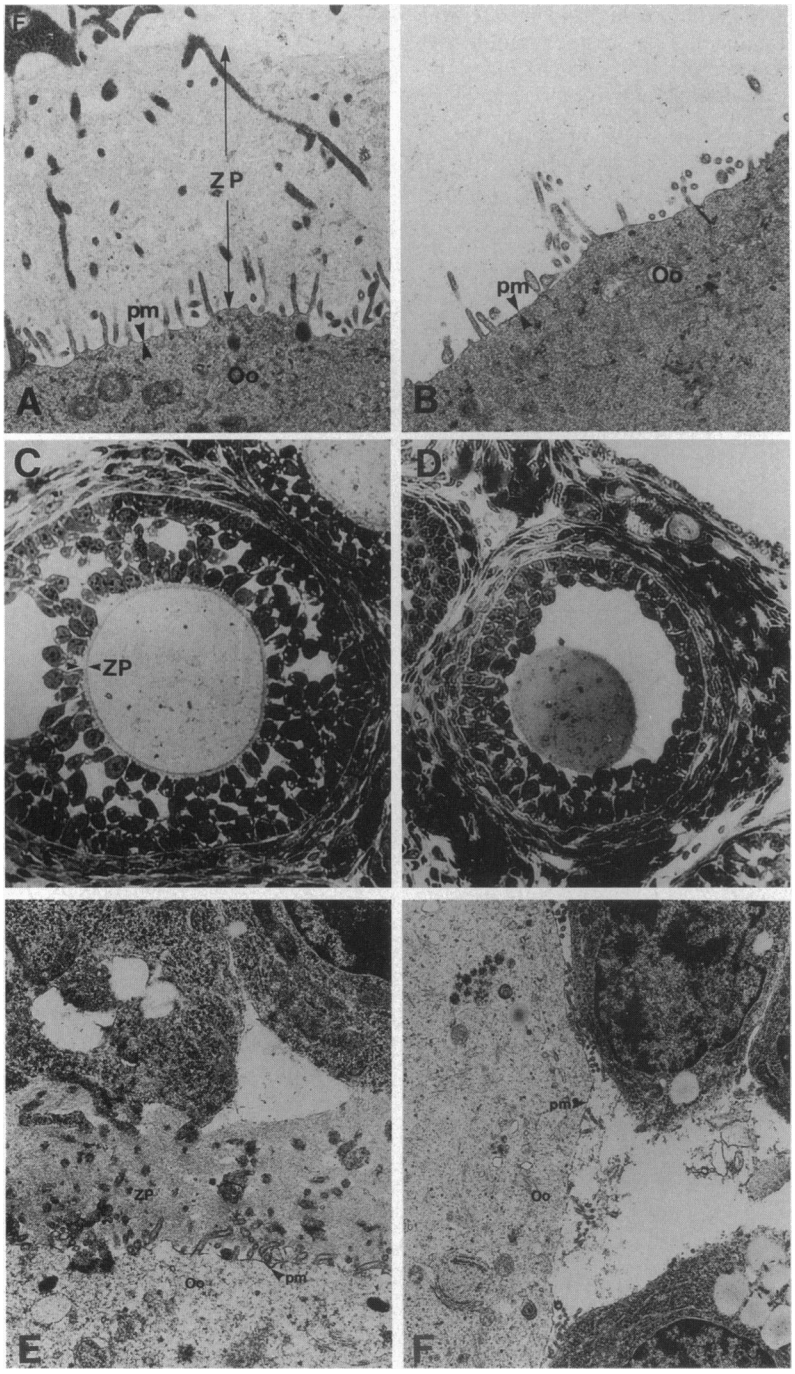

FIG. 3. Light and electron micrographs of growing oocytes and ovarian follicles in *mZP3*$^{-/-}$ and wild-type juvenile and adult female mice. (*A*) Electron micrograph of a growing oocyte in the ovary of a 17-day-old wild-type mouse. Shown is an oocyte (Oo; ≈70 μm in diameter), with its thick ZP, and some surrounding follicle cells (F). Note the large processes extending from the follicle cells through the ZP toward the oocyte. (*B*) Electron micrograph of a growing oocyte (Oo; ≈65 μm in diameter) in the ovary of a 17-day-old *mZP3*$^{-/-}$ mouse. Note both the complete absence of a ZP and the lack of follicle cells in close proximity to the oolemma (pm). (*C*) Light micrograph of an ovarian follicle in a ≈6 week-old (adult) wild-type mouse. Note that the oocyte has a ZP and is completely surrounded by follicle cells within the antral follicle. (*D*) Light micrograph of an ovarian follicle in a ≈6 week-old (adult) *mZP3*$^{-/-}$ mouse. Note that the oocyte does not have a ZP and is not completely surrounded by follicle cells within the antral follicle. (*E*) Electron micrograph of a portion of an ovarian follicle in a ≈6 week-old (adult) wild-type mouse. Note the thick ZP and close apposition of the follicle cells that are sending processes into the ZP. (*F*) Electron micrograph of a portion of an ovarian follicle in a ≈6 week-old (adult) *mZP3*$^{-/-}$ mouse. Note the complete absence of a ZP and the apparent absence of processes extending from the follicle cells to the oocyte. (*A* and *B*, ×≈7000; *C* and *D*, ×≈410; *E* and *F*, ×≈3500.)

follicle cells themselves are interconnected by gap junctions, this means that the oocyte is both metabolically and ionically coupled to them (38). The situation in female *mZP3*$^{-/-}$ mice differs from wild-type mice in that the oocyte is not completely surrounded by follicle cells. In addition, in the absence of a ZP there appear to be fewer follicle cell processes contacting the growing oocyte. It will be of considerable interest to examine the relationship between oocyte and follicle development in female *mZP3*$^{-/-}$ mice using various biochemical and molecular approaches.

In summary, we have created mice homozygous for a null mutation in the *mZP3* gene by targeted mutagenesis. Homozygous mutant female mice produce growing oocytes and unfertilized eggs, but they do not have a ZP. Furthermore,

although the mutant mice ovulate and exhibit normal reproductive behavior, they are infertile. These mice should be extremely valuable for investigating ZP structure and function, as well as for studies of oocyte-follicle cell interactions.

We are very grateful to Susan Abbondanzo who often guided us through some of the vagaries of targeted mutagenesis.

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