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Invited Review

Ovarian development in mice bearing homozygous or heterozygous null mutations in zona pellucida glycoprotein gene *mZP3*

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Summary. The plasma membrane of all mammalian eggs is surrounded by a thick extracellular coat, the *zona pellucida* (ZP), whose paramount function is to regulate species-specific fertilization. The mouse egg ZP is composed of only three glycoproteins, mZP1-3, that are synthesized and secreted exclusively by oocytes during their 2-3 week growth phase. Disruption of the *mZP3* gene by targeted mutagenesis in embryonic stem (ES) cells yields mice heterozygous ($mZP3^{+/-}$) or homozygous ($mZP3^{-/-}$) for the null mutation. As expected, male mice bearing the null mutation are indistinguishable from wild-type males with respect to viability and fertility. Female $mZP3^{+/-}$ mice are as fertile as wild-type animals, but their eggs have a thin ZP (~2.7 μm thick) as compared to the ZP (~6.2 μm thick) of eggs from wild-type animals. On the other hand, female $mZP3^{-/-}$ mice are infertile and their eggs lack a ZP. The infertility apparently is due to the lack of a sufficient number of eggs in oviducts of superovulated $mZP3^{-/-}$ females. Light micrographs reveal that development of ovarian follicles is often retarded in $mZP3^{-/-}$ mice as compared to wild-type animals. This is manifested as reduced ovarian weights, reduced numbers of Graafian follicles, and reduced numbers of fully-grown oocytes in $mZP3^{-/-}$ females. It seems likely that the pleiotropic effects of the homozygous null mutation on ovarian development may be due, at least in part, to disruption of intercellular communication between growing oocytes and their surrounding follicle cells.

Key words: Fertilization, Zona pellucida, Null mutation, Mouse

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Introduction

The plasma membrane of all mammalian eggs is surrounded by a relatively thick extracellular coat, called the *zona pellucida* (ZP) (Fig. 1; Gwatkin, 1977; Dietl, 1989; Wassarman and Albertini, 1994; Yanagimachi, 1994). The ZP forms a viscous border between the oolemma and the innermost layer of follicle cells (*corona radiata*). Cellular processes extend from the innermost follicle cells, through the ZP, and form gap junctions with egg plasma membrane. The ZP performs functions identical to those performed by the jelly coat and vitelline envelope of eggs from non-mammalian species (e.g., echinoderms and amphibia). In particular, it is the site of species-specific sperm receptors and acrosome reaction-inducers that function in vivo during fertilization of the egg (Wassarman, 1990, 1995; Snell and White, 1996; Shalgi and Raz, 1997). Removal of the ZP eliminates the barrier to *in vitro* fertilization of eggs by sperm from different species.

The mouse egg ZP is about 6.2 (±1.9) μm thick, contains about 3.5 (±0.5) ng of protein, and stains positively for glycoprotein (Wassarman, 1988, 1997). It consists of three glycoproteins, mZP1 (~200 kDa Mr; dimer), mZP2 (~120 kDa Mr; monomer), and mZP3 (~83 kDa Mr; monomer), that combine to form an extensive network of long, crosslinked filaments by using non-covalent bonds (Greve and Wassarman, 1985; Wassarman and Mortillo, 1991). Each of the glycoproteins is heterogeneous with respect to Mr due to heterogeneous glycosylation of a unique polypeptide (polypeptide Mr - mZP1, 68 kDa; mZP2, 77 kDa; mZP3, 44 kDa) with both asparagine- (N-) linked (complex-type) and serine/threonine- (O-) linked oligosaccharides. The oligosaccharides are both sulfated and sialylated (Noguchi and Nakano, 1993; Liu et al., 1997), which contributes to the Mr heterogeneity and makes all three glycoproteins relatively acidic.

In mice, free-swimming, acrosome-intact sperm

recognize and bind to specific O-linked oligosaccharides located on the carboxy-terminal portion of mZP3 (a region encoded by *mZP3* exon-7) (Bleil and Wassarman, 1980; Florman and Wassarman, 1985; Rosiere and Wassarman, 1992; Kinloch et al., 1995; Litscher and Wassarman, 1996). Each acrosome-intact sperm head possesses tens-of-thousands of sites that are capable of binding to mZP3 in the ZP (Bleil and Wassarman, 1986; Mortillo and Wassarman, 1991). Once bound to mZP3 by plasma membrane overlying their head, sperm undergo the acrosome reaction (cellular exocytosis), lose plasma membrane and outer acrosomal membrane, and remain associated with the ZP by binding to mZP2 by their inner acrosomal membrane (Bleil and Wassarman, 1983; Bleil et al., 1988; Ward and Kopf, 1993). Thus, during fertilization mZP3 serves as a primary sperm receptor and acrosome reaction-inducer, while mZP2 serves as a secondary sperm receptor (Wassarman, 1990).

Targeted mutagenesis of the *mZP3* gene, by homologous recombination in embryonic stem (ES) cells (Wassarman and DePamphilis, 1993; Hogan et al., 1994), results in production of homozygous null mutant females ($mZP3^{-/-}$) that are infertile and whose eggs lack a ZP (Liu et al., 1996; Rankin et al., 1996; Wassarman et al., 1996) and heterozygous null mutant females ($mZP3^{+/-}$) that are fertile, but whose eggs have a thin ZP (Wassarman et al., 1997). Here, we describe the production and phenotype of these animals, and speculate on the reasons for the mutation's effects on ovarian development and fertility.

Production of null mutant mice

mZP3 null mutant mice were produced (Liu et al., 1995, 1996) using homologous recombination in ES cells by following standard gene targeting procedures (Wassarman and DePamphilis, 1993; Hogan et al., 1994). The targeting vector was constructed such that part of *mZP3* exon-2 and exon-3, and all of *mZP3* intron-2, were replaced by a positive selection marker (pGKneo) (Fig. 2). Six positive ES cell lines were injected into mouse blastocysts and all were transmitted through the germ line. Heterozygous ($mZP3^{+/-}$) and homozygous ($mZP3^{-/-}$) female mutant mice were identified by polymerase chain reaction (PCR) assays (Fig. 3) and Southern blotting, and mZP3 messenger-RNA and glycoprotein levels were assessed by Northern and Western blotting, respectively. Whereas, mZP3 messenger-RNA and glycoprotein were present in ovaries excised from $mZP3^{+/+}$ and $mZP3^{+/-}$ mice, they were not present in ovaries excised from $mZP3^{-/-}$ mice. Despite these differences, $mZP3^{-/-}$ female mice were indistinguishable in appearance from wild-type and

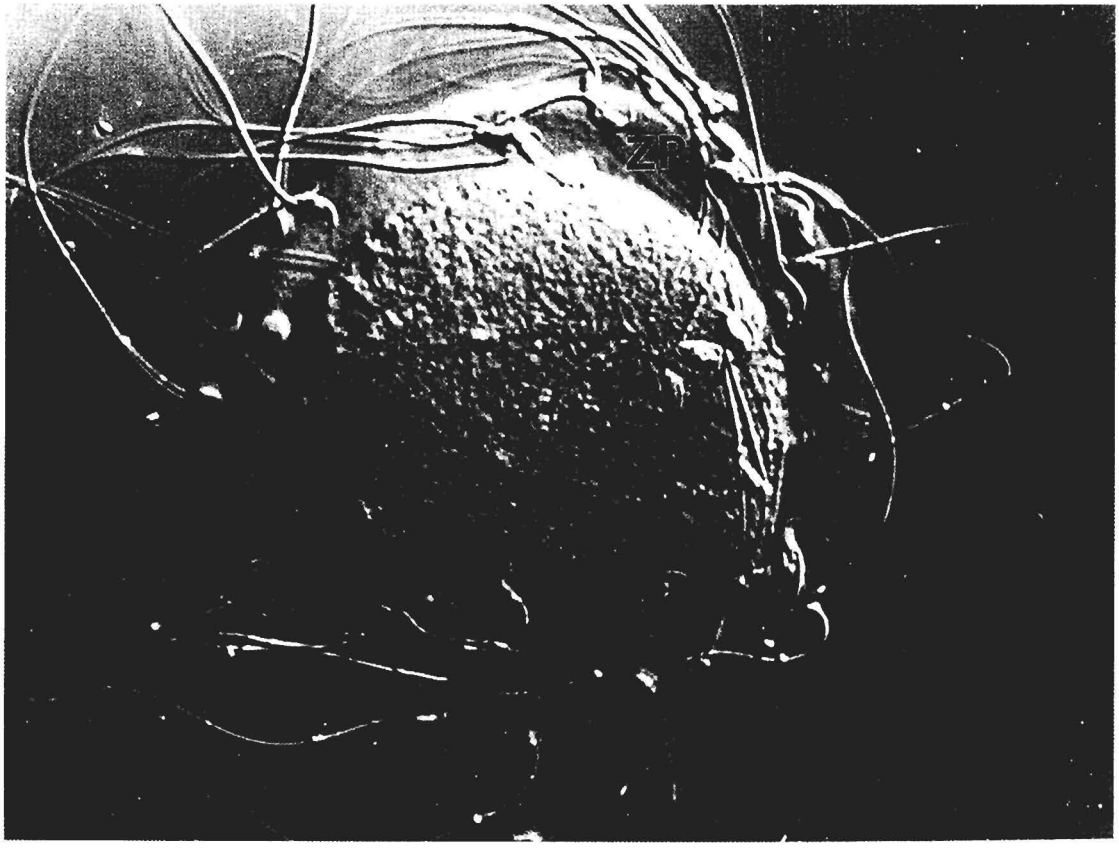


Fig. 1. Light micrograph of free-swimming mouse sperm bound by their heads to the ZP of an ovulated mouse egg *in vitro*. The micrograph was taken by using Nomarski differential interference contrast (DIC) microscopy. zp: *zona pellucida*. x 1,000

heterozygous littermates, and exhibited normal growth and development.

Phenotype of heterozygous null mutant mice

Oocytes and eggs isolated from females heterozygous for the *mZP3* mutation ($mZP3^{+/-}$) have a ZP (Liu et al., 1996). On the other hand, the ZP is less than one-half the thickness (~2.7 μm) of the ZP from wild-type mice (~6.2 μm) (Fig. 4; Wassarman et al., 1997). Immunostaining of purified ZP on Western gels permitted an estimate to be made of the relative amounts of mZP3 and mZP2 present in the ZP of oocytes from $mZP3^{+/+}$ and $mZP3^{+/-}$ mice. The ZP from $mZP3^{+/-}$ mice contained, on average, 55±15% of the mZP3 and 44±8% of the mZP2 present in the ZP of oocytes from wild-type mice; a result quite consistent with the observed widths of the ZP. These results strongly suggest that, when a single *mZP3* allele is present, approximately one-half the wild-type amount of mZP3 and approximately one-half the wild-type amount of mZP2 is assembled into a ZP. These results are consistent with the current model for ZP structure.

Despite the presence of a thinner than usual ZP surrounding their eggs, $mZP3^{+/-}$ females reproduced normally (Wassarman et al., 1997). We found no differences in reproduction (e.g., presence of copulation plugs, rates of pregnancy, and litter size) between $mZP3^{+/-}$ and $mZP3^{+/+}$ females. For example, in matings of $mZP3^{-/-}$ males with $mZP3^{+/-}$ females, the average litter contained 7.9 pups (ave. of 8 litters); in matings of $mZP3^{-/-}$ males with $mZP3^{+/+}$ females, the average litter contained 7.8 pups (ave. of 8 litters). This is to be contrasted with matings of $mZP3^{-/-}$ females with $mZP3^{-/-}$ or $mZP3^{+/-}$ males that resulted in no pregnancies. Thus, despite the presence of a relatively thin ZP on eggs from $mZP3^{+/-}$ females, apparently reproduction is not affected.

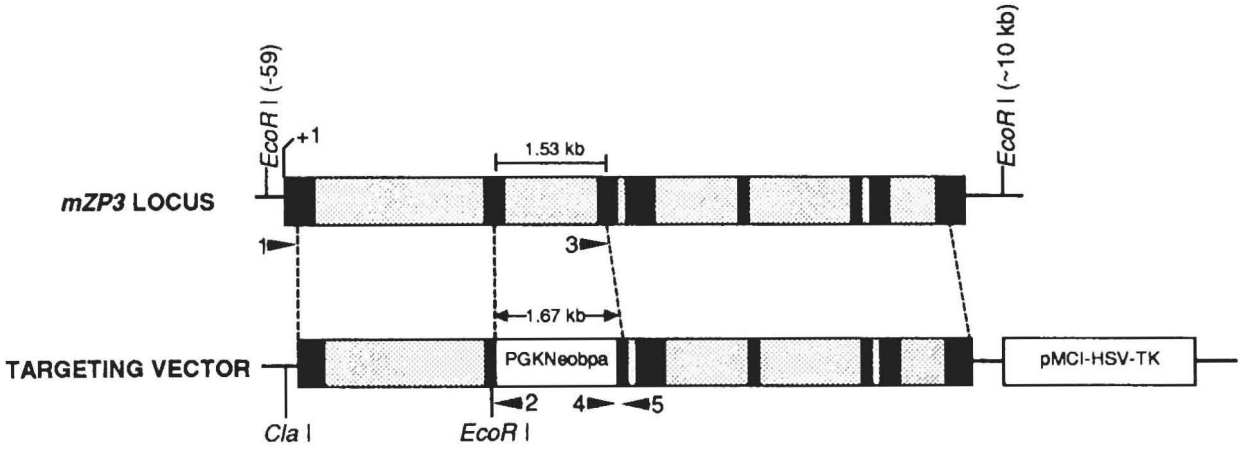


Fig. 2. Schematic representation of the *mZP3* locus and the targeting vector used to produce homozygous and heterozygous null mutant mice. Black boxes represent exons, stippled boxes represent introns, and lines represent upstream and downstream flanking sequences of the *mZP3* gene. In the targeting 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 EcoRI and ClaI 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. For details of the methodology, see Liu et al., 1996.

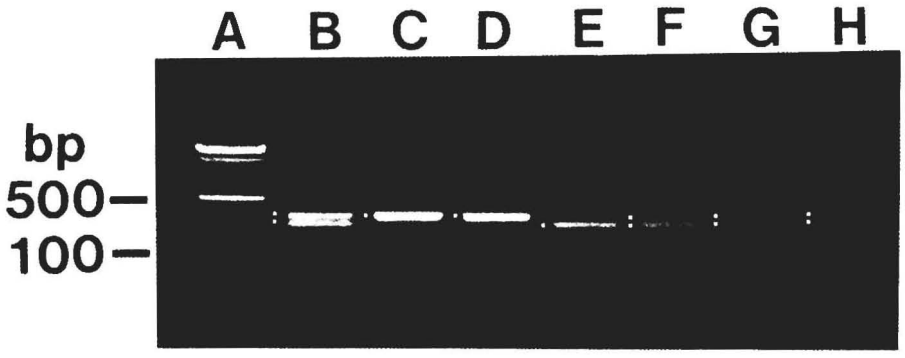


Fig. 3. Identification of wild-type, heterozygous mutant, and homozygous mutant female mice by PCR analysis of tail DNA. PCR conditions and primers used are described in Liu et al., 1996. Shown is a gel on which seven samples of amplified mouse DNA (lanes B-H) and DNA markers (lane A) were run. The pattern of DNA bands indicates that the tail DNA came from wild-type (lane E; 319-base pair band), homozygous mutant (lanes C and D; 410-base pair band), and heterozygous mutant (lanes B, F, G, and H; 319- and 410-base pair bands) female mice. Amplified bands are indicated by white dots. The DNA markers (lane A) represent a 100-base pair ladder (the 100-base pair and 500-base pair bands are indicated). For details of the methodology see Liu et al., 1996 and Wassarman et al., 1997.

Phenotype of homozygous null mutant mice

The external phenotypes of *mZP3*$^{-/-}$ and *mZP3*$^{+/+}$ female mice are identical and homozygous null mutant females grow at the same rate as wild-type females (Liu et al., 1996). However, growing oocytes in *mZP3*$^{-/-}$ mice do not have a ZP (Fig. 5) and there is a significant difference in the size of their ovaries as compared to

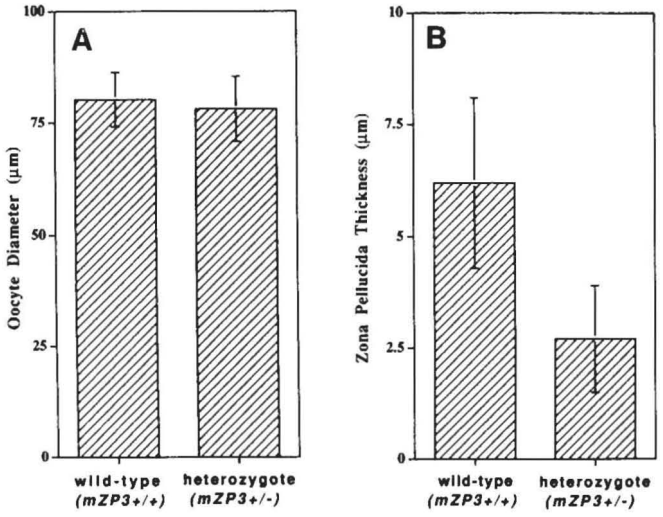


Fig. 4. Estimation of the diameter of oocytes and the thickness of the ZP of oocytes recovered from ovaries of heterozygous mutant and wild-type female mice. **A.** Shown is the diameter of fully-grown oocytes (μm± s.d.) recovered from *mZP3*$^{+/+}$ and *mZP3*$^{+/-}$ mice. The average diameter of oocytes from wild-type mice is 80±6 μm. The average diameter of oocytes from heterozygous mutant mice is 78±7 μm. **B.** Shown is the thickness of the ZP (μm±s.d.) of fully-grown oocytes recovered from *mZP3*$^{+/+}$ and *mZP3*$^{+/-}$ mice. The average thickness of the ZP of oocytes from wild-type mice is 6.2±1.9 μm. The average thickness of the ZP of oocytes from heterozygous mutant mice is 2.7±1.2 μm. For details, see Wassarman et al., 1997.

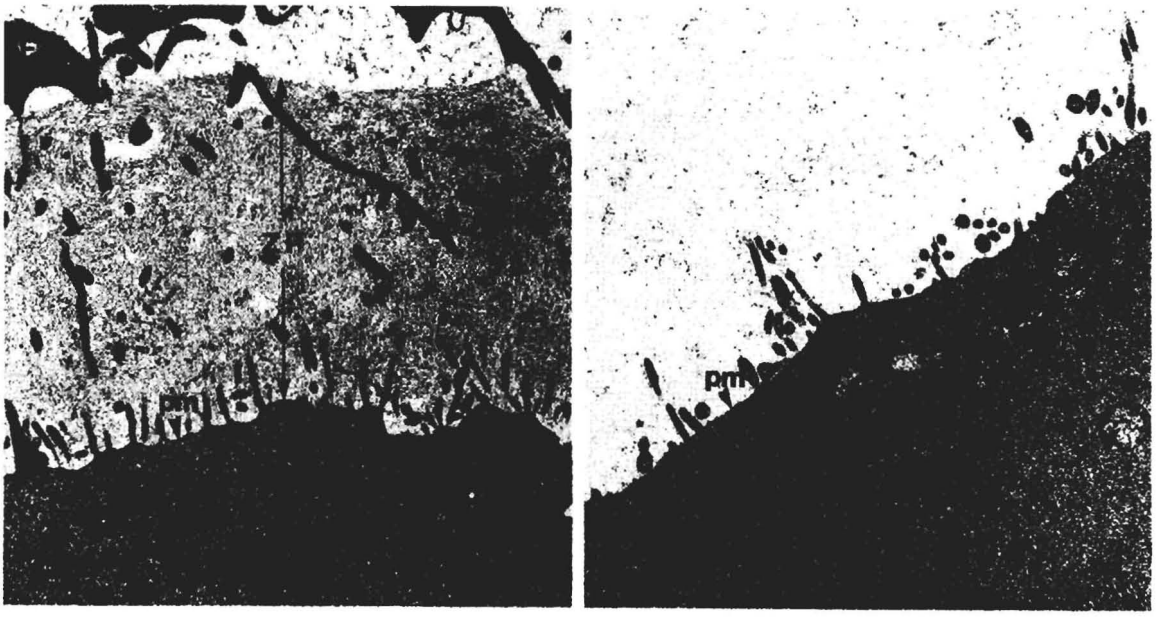


Fig. 5. Electron micrographs of growing oocytes in juvenile wild-type and homozygous null mutant female mice. **A.** Electron micrograph of a growing oocyte in the ovary of a 17 day-old wild-type mouse. Shown is an oocyte (Oo; ~70 μm in diameter), with its thick ZP, and some surrounding follicle cells (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). For details see Liu et al., 1996. x 12,000

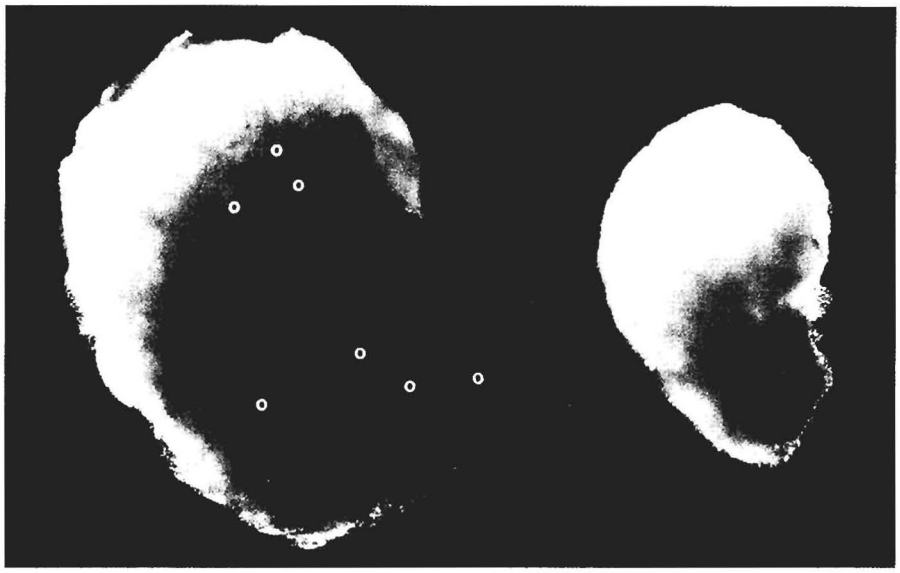
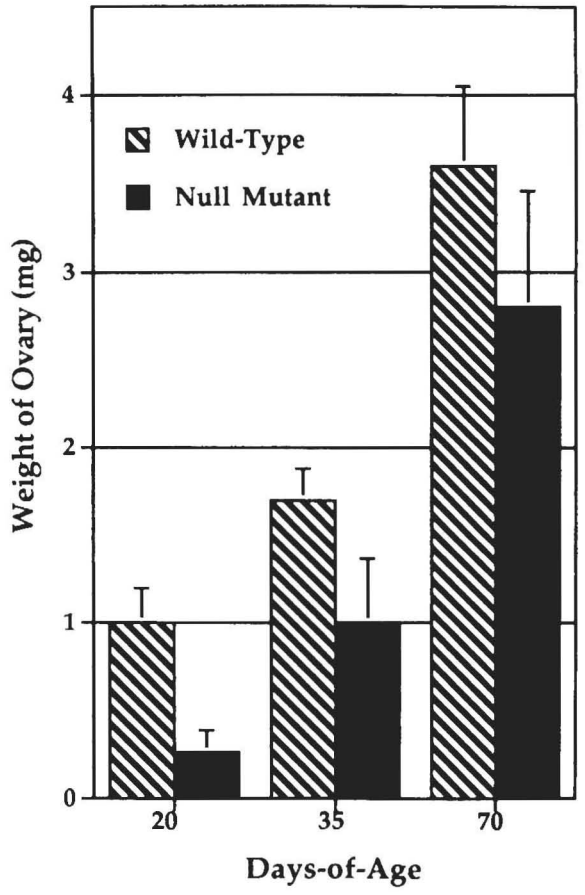


Fig. 6. Photomicrograph of ovaries from juvenile wild-type and homozygous null mutant female mice. Juvenile animals, 20 days-of-age, were sacrificed and ovaries were excised, placed in culture medium, and photographed by using a Zeiss IM35 inverted microscope. Shown are ovaries from a (left) wild-type (*mZP3*$^{+/+}$) and a (right) homozygous null mutant (*mZP3*$^{-/-}$) animal. **Left:** Note the large number of antral follicles on the surface of the ovary excised from the wild-type mouse (some are indicated by white circles). **Right:** Few, if any, antral follicles are present on the surface of the ovary excised from the homozygous null mutant mouse. x 25

wild-type mice (Liu et al., 1996). Ovaries from wild-type animals are always larger than ovaries from *mZP3*$^{-/-}$ animals, with the largest difference (~4-fold) seen in juvenile (≤21 days-of-age) mice (Fig. 6). The ratio of average ovarian weights (wild-type:null mutant) for 20-, 35-, and 70-day-old mice changes from about 4, to 1.7, to 1.3, respectively (Fig. 7). This phenotype apparently is due to retarded follicular development in *mZP3*$^{-/-}$ mice which is seen most clearly in juvenile females. Most of the growing oocytes in ovaries from null mutant mice are misshapen and are not intimately associated with surrounding follicle cells (Fig. 8). In addition, the ovaries contain few, if any, Graafian follicles. In general, oocytes from *mZP3*$^{-/-}$ mice do not have a ZP and the overall organization of follicular components is altered in *mZP3*$^{-/-}$ mice as compared to wild-type mice.



Interestingly, female *mZP3*$^{-/-}$ mice are infertile and the infertility apparently is related to the complete absence of a ZP on ovulated eggs (Liu et al., 1996). 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 masses. In this connection, the number of oocytes obtained from ovaries of null mutant females was only about 15% the number obtained from ovaries of wild-type mice. These findings are consistent with the observation that *mZP3*$^{-/-}$ oocytes are not intimately associated with surrounding follicle cells in the ovary. Of course, it is possible that most *mZP3*$^{-/-}$ oocytes that are ovulated cannot withstand the force of ovulation and lyse due to the absence of a ZP.

Fig. 7. Estimation of the weight of ovaries excised from juvenile and adult wild-type and homozygous null mutant female mice. Shown are the weights of ovaries excised from the wild-type (hatched bars; *mZP3*$^{+/+}$) and mutant (solid black bars; *mZP3*$^{-/-}$) animals in milligrams (mg±s.d.) as a function of the age of the mice (20, 35, and 70 days-of-age) (see Materials and Methods). In each case, the weights of 16-22 ovaries were determined. It should be noted that, in the case of ovaries from 20-day-old mice, each measurement was made with 2 ovaries and the result was divided by 2.

Discussion of null mutant phenotypes

The observations presented above emphasize the importance of ZP assembly during follicle development. In the complete absence of a ZP (*mZP3*$^{-/-}$), both oocyte growth and follicle cell proliferation appear to be deleteriously affected and females are infertile (Liu et al., 1996; Wassarman et al., 1996). These findings suggest that ZP formation is not only critical to the process of fertilization, but also to the final stages of development of the unfertilized egg from a non-growing oocyte during each reproductive cycle.

The thickness of the egg ZP varies greatly from one mammalian species to another (from ~2 μm to ~25 μm; Dunbar et al., 1991). Consequently, it is not surprising that a thinner than normal ZP on heterozygous null mutant mouse eggs (2.7 μm vs. 6.2 μm) can support oocyte growth, follicle cell proliferation, fertilization, and preimplantation development (Liu et al., 1996; Wassarman et al., 1996, 1997). The presence of a single *mZP3* allele allows the synthesis and assembly of about one-half the normal complement of mZP3 (55±15%) and the assembly of about one-half the normal complement of mZP2 (44±8%). Since mZP2 appears to be present at wild-type levels in *mZP3*$^{+/-}$ mice, this suggests that only a portion of the mZP2 available is assembled into a ZP. This is consistent with current views of ZP assembly in which mZP2-mZP3 dimers are polymerized into long, interconnected filaments (Greve and Wassarman, 1985; Wassarman and Mortillo, 1991). In the case of *mZP3*$^{+/-}$ mice, since only one-half of the normal complement of mZP3 is available, only one-half of the mZP2 complement can be accommodated for ZP filament formation during oocyte growth. The fate of the

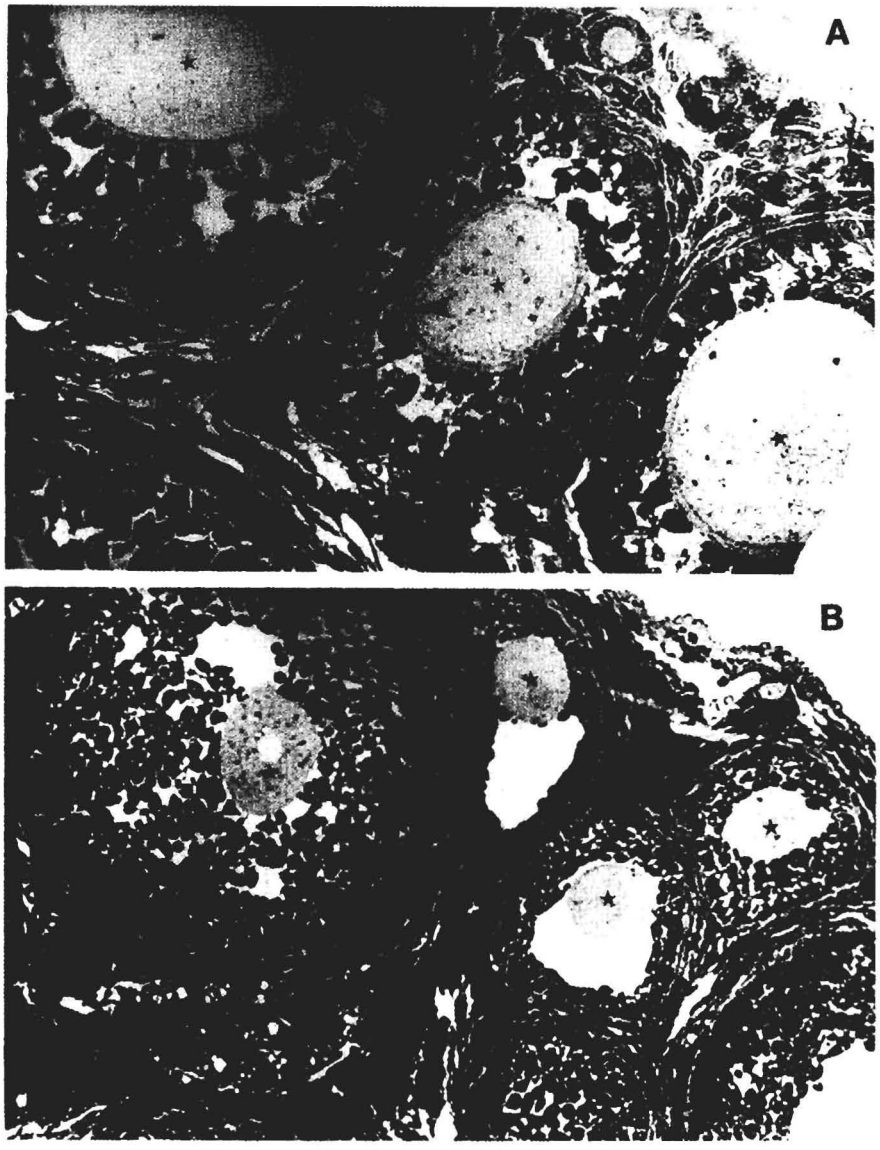


Fig. 8. Light micrographs of ovarian sections taken from adult wild-type and homozygous null mutant female mice. Shown are ovarian sections taken from 35-day-old wild-type (Panel A; *mZP3*$^{+/+}$) and homozygous null mutant (Panel B; *mZP3*$^{-/-}$) mice (see Materials and Methods). **A.** An arrowhead indicates the position of the ZP and asterisks indicate the positions of oocytes. Note that the oocytes are in very close proximity with the surrounding follicle cells. **B.** Asterisks indicate the positions of oocytes. Note that the oocytes do not have a ZP and that the oocytes are only loosely associated with the follicle cells as compared to Panel A. x 900

remaining egg mZP2 is unclear at the moment, but is under investigation. It will be of considerable interest to determine whether or not the remaining pool of mZP2 is secreted, remains stored within the growing oocyte, or is turned-over by proteolysis.

Oocytes and eggs from *mZP3*$^{-/-}$ females lack a ZP and the females are infertile (Liu et al., 1996; Wassarman et al., 1996). The former finding is not surprising since mZP3 is an important structural component of the ZP. The phenotype of ovaries from homozygous null mutant animals is complex since several different parameters are affected to varying extents in different animals. For example, although *mZP3*$^{-/-}$ oocytes grow, they are frequently misshapen and do not appear to be as healthy as oocytes from wild-type mice. Similarly, *mZP3*$^{-/-}$ follicles develop to varying extents, but overall appear to be retarded in their development as compared to wild-type follicles. The latter is most pronounced in juvenile mice, where the ovaries are about one-quarter the weight of ovaries from wild-type mice, apparently due to retarded follicle development. This is most demonstrable since the ovarian stroma and vasculature (Duke, 1978; Ellinwood et al., 1978) are incomplete in prepubertal juvenile mice. These observations strongly suggest that interactions between the growing oocyte and proliferating follicle cells are partially disrupted in ovaries of *mZP3*$^{-/-}$ mice.

If the ZP normally serves as a "glue" with which to stabilize gap junctions formed between oocytes and their innermost follicle cells (*corona radiata*), then the absence of a ZP could severely affect oocyte-follicle cell communication. Gap junctions are aggregations of intercellular channels composed of connexins, a family of related proteins that connect adjacent cells and facilitate electrical and metabolic coupling between cells (Goodenough et al., 1996; Sosinsky, 1996). Several studies have revealed the presence of gap junctions between mammalian oocytes and surrounding follicle cells (Anderson and Albertini, 1976; Amsterdam et al., 1976; Gilula et al., 1978) at sites where follicle cell processes traverse the ZP and contact the oolemma. These gap junctions apparently play a vital role in oocyte growth, both *in vivo* and *in vitro*, and in meiotic maturation at the time of ovulation (Brower and Schultz, 1982; Eppig, 1982, 1985, 1994; Schultz, 1986).

What happens when gap junctions cannot form between mouse oocytes and follicle cells? Recently, this question was addressed directly by producing connexin-37 deficient female mice (Cx37$^{-/-}$) which lack recognizable gap junctions between oocytes and follicle cells (Simon et al., 1997). Such females fail to produce Graafian follicles and do not ovulate unfertilized eggs; that is, Cx37$^{-/-}$ females are infertile. Our observations with *mZP3*$^{-/-}$ females mirror somewhat those obtained with Cx37$^{-/-}$ mice and could be due, at least in part, to reduced intercellular communication between growing oocytes and follicle cells. This can be tested experimentally and is currently under investigation in our laboratory.

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