Mutant female mice carrying a single *mZP3* allele produce eggs with a thin zona pellucida, but reproduce normally

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SUMMARY

The mouse egg zona pellucida (ZP) is composed of three glycoproteins, called mZP1–3. Disruption of the *mZP3* gene by targeted mutagenesis yields mice that are homozygous ($mZP3^{-/-}$) for the null mutation; although the mutant mice are viable, females are infertile and their eggs lack a ZP. On the other hand, females heterozygous ($mZP3^{+/-}$) for the mutation are fertile and their eggs have a ZP. Here, we examined fully grown oocytes from $mZP3^{+/-}$ females and found that, although they have a ZP, it is less than half the width (~ 2.7 μm; volume, ~ 56 pl) of the ZP of oocytes from wild-type ($mZP3^{+/+}$) mice (~ 6.2 μm; volume, ~ 145 pl). Oocyte ZP were purified from ovarian homogenates by gradient centrifugation. 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. We found that the ZP from $mZP3^{+/-}$ mice contained, on average, $55 \pm 15\%$ of the mZP3 and $44 \pm 8\%$ of the mZP2 present in the ZP of $mZP3^{+/+}$ mice; a result quite consistent with the observed widths and calculated volumes of the ZP. Despite the presence of a relatively thin ZP surrounding their eggs, reproduction of female $mZP3^{+/-}$ mice was indistinguishable from female $mZP3^{+/+}$ mice. These results strongly suggest that, when a single *mZP3* allele is present, approximately half the wild-type amount of mZP3 and approximately half the wild-type amount of mZP2 is assembled into a ZP. While this produces a relatively thin ZP, it apparently has no affect on reproduction. Furthermore, these results are consistent with the current molecular model for ZP structure.

1. INTRODUCTION

All mammalian eggs are surrounded by an extracellular coat, called the zona pellucida (ZP), that is composed of only a few glycoproteins (Wassarman 1988; Dietl 1989). The ZP plays several important roles during oogenesis, fertilization and preimplantation development (Dietl 1989; Yanagimachi 1994). Perhaps paramount among these is the role of the ZP during binding of free-swimming sperm to ovulated eggs (Wassarman 1995; Snell & White 1996). It is well documented that the ZP contains species-specific sperm receptors that, together with complementary egg-binding proteins on sperm, restrict binding of sperm from heterologous species to eggs (Wassarman 1987, 1995; Yanagimachi 1994; Snell & White 1996). In addition, following fertilization the ZP undergoes changes that prevent the binding of sperm from the homologous species to the fertilized egg (Wassarman 1987; Yanagimachi 1994).

The mouse egg ZP is constructed of three glycoproteins, called mZP1–3 (Wassarman *et al.* 1985; Wassarman 1988, 1995). The ZP consists of long filaments, composed of mZP2 and mZP3 and interconnected to each other by mZP1 (Greve & Wassarman 1985; Wassarman 1988; Wassarman & Mortillo 1991). mZP3 serves as a species-specific receptor or ligand for free-swimming sperm during fertilization (Bleil & Wassarman 1980; Florman & Wassarman 1985; Wassarman *et al.* 1985; Wassarman 1990, 1995; Wassarman & Litscher 1995). Acrosome-intact sperm bind to specific serine/threonine- (O-) linked oligosaccharides present on mZP3 and then undergo the acrosome reaction (i.e. cellular exocytosis) in response to binding to mZP3.

Recently, we (Liu *et al.* 1995, 1996; Wassarman *et al.* 1996) and others (Rankin *et al.* 1996) disrupted the *mZP3* gene by targeted mutagenesis using homologous recombination in mouse embryonic stem (ES) cells. Although female homozygous ($mZP3^{-/-}$) null mutant mice were indistinguishable in appearance from wild-type ($mZP3^{+/+}$) and heterozygous ($mZP3^{+/-}$) littermates, and exhibited normal growth and development, their eggs lacked a ZP and the animals were infertile. The infertility apparently is due to the failure of eggs lacking a ZP to reach the oviduct during ovulation. On average, less than 10% of the ovulated eggs found in oviducts of wild-type females are found in oviducts of $mZP3^{-/-}$ females.

During our initial studies with *mZP3* null mutant mice (Liu *et al.* 1996) we noted that although oocytes and eggs from $mZP3^{+/-}$ females had a ZP, it appeared

to be thinner than the wild-type ZP. Here, we confirmed that, indeed, there is a considerable difference in the width of the ZP from heterozygous mutant and wild-type mice. This difference is also reflected in the amounts of mZP3 and mZP2 found in the ZP of oocytes from $mZP3^{+/-}$ mice. While this difference apparently does not affect reproduction, it provides some insight into the structure of the ZP and its assembly during oogenesis.

2. MATERIALS AND METHODS

(*a*) *Production and analysis of mice heterozygous for the mZP3 null mutation*

Vector construction for targeted mutagenesis of the *mZP3* gene, transfection, selection and screening of mouse embryonic stem (ES) cells, and production of mice homozygous for the null mutation have been described (Liu *et al*. 1996). Homozygous ($mZP3^{-/-}$) or heterozygous ($mZP3^{+/-}$) males were mated with wild-type ($mZP3^{+/+}$) or heterozygous ($mZP3^{+/-}$) females, and offspring were analysed by PCR using tail DNA to identify wild-type ($mZP3^{+/+}$) and heterozygous ($mZP3^{+/-}$) mutant female mice. The same three PCR primers and the same PCR conditions as previously described (Liu *et al*. 1996) were used here. DNA from heterozygous and wild-type mice results in amplification of 319-bp and 410-bp bands and a 319-bp band, respectively (see figure 1, Liu *et al*. (1996)).

(*b*) *Purification of mZP3 and mZP2 from ovaries of heterozygous and wild-type mice*

Ovaries were excised from sexually mature $mZP3^{+/-}$ and $mZP3^{+/+}$ mice (*ca*. five weeks of age), homogenized, and were then either used directly or subjected to gradient centrifugation through Percoll (Sigma) to isolate ZP, as previously described (Bleil & Wassarman 1986; Bleil *et al*. 1988). mZP3 and mZP2 were purified from ZP preparations by HPLC on a size-exclusion column (Bio-Sil, SEC-250), as previously described (Bleil & Wassarman 1986; Bleil *et al*. 1988).

(*c*) *Western blot analyses of mZP3 and mZP2*

Western blot analysis was carried out with either ovarian homogenates or isolated ZP preparations subjected to SDS/PAGE, transferred to nitrocellulose, and probed with a polyclonal rabbit anti-mZP3 or anti-mZP2 IgG (Pocono Farms) followed by an alkaline phosphatase conjugated goat anti-rabbit IgG (Bio-Rad) and NBT/BCIP (GIBCO/BRL), as previously described (Liu *et al*. 1995). In most cases, several known amounts of purified mZP3 or mZP2 were applied to the gels in order to construct a standard curve. Dried blots were scanned using a personal densitometer (Molecular Dynamics) equipped with Image Quant (version 2), which permitted determination of relative amounts of mZP3 and mZP2.

(*d*) *Measurements of ZP of fully-grown oocytes from heterozygous and wild-type mice*

Fully grown oocytes were obtained by poking ovaries excised from sexually mature female mice with fine steel needles and were cultured, as previously described (Rafferty 1970; Schultz *et al*. 1979). Oocyte diameters (exclusive of the ZP) and ZP widths were measured with an ocular micrometer attached to a Zeiss IM35 inverted microscope and corresponding volumes were calculated by assuming the oocytes to be perfect spheres. Eggs were obtained from superovulated females injected with pregnant mare's serum (PMS, 5 IU; Sigma) and human chorionic gonadotropin (hCG, 5 IU; Sigma) 48 h later (Rafferty 1970; Schultz *et al*. 1979). Oocytes and eggs were cultured *in vitro*, essentially as previously described (Bleil & Wassarman 1980; Florman & Wassarman 1985). Ovulated eggs were fixed with 1% formaldehyde prior to photomicroscopy.

3. RESULTS

(*a*) *Identification of* $\mathbf{mZP3^{+/-}}$ *mice*

mZP3 null mutant mice ($mZP3^{-/-}$) were produced (Liu *et al*. 1995, 1996) using homologous recombination in ES cells by following standard gene targeting procedures (Wassarman & DePamphilis 1993; Hogan *et al*. 1994). Wild-type or heterozygous females were mated with heterozygous or homozygous null mutant males, and heterozygous mutant female pups ($mZP3^{+/-}$) were identified by PCR using tail DNA. As seen in figure 1, PCR of DNA from $mZP3^{+/-}$ mice yielded a doublet of 319-bp and 410-bp bands on gels; indicative of the presence of only a single *mZP3* allele. Wild-type and homozygous mutant females yielded 319-bp and 410-bp bands, respectively.

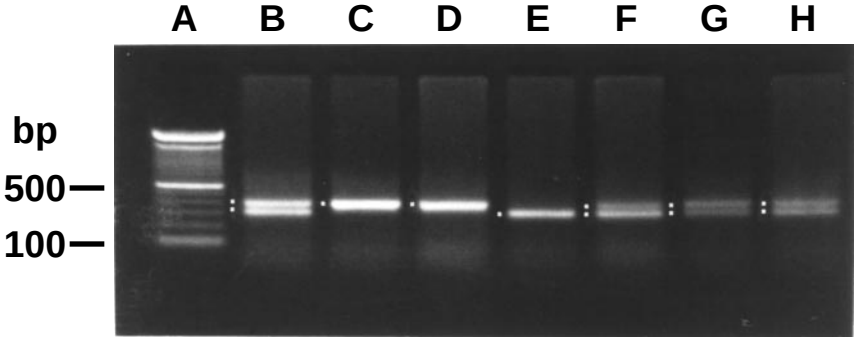

Figure 1. Identification of wild-type, heterozygous mutant, and homozygous mutant female mice by PCR analysis of tail DNA. PCR conditions and the primers used are described in §2. Shown is a gel on which seven samples of amplified mouse DNA (lanes B–H) and DNA markers (lane A) were run. The pattern of 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-bp and 500-bp bands are indicated).

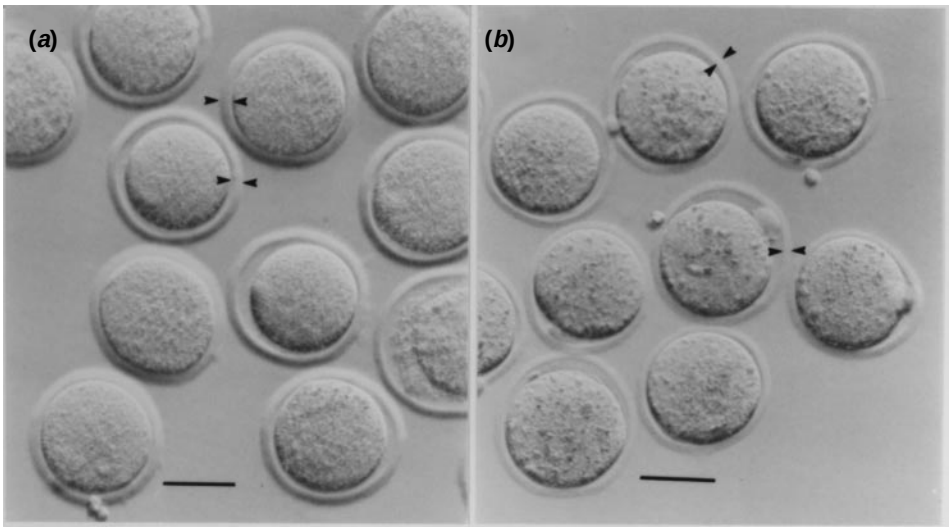

Figure 2. Photomicrographs of ovulated eggs from heterozygous mutant and wild-type female mice. Adult animals were superovulated and ovulated eggs were recovered and fixed, as described in §2. Shown are eggs from superovulated $mZP3^{+/+}$ (*a*) and $mZP3^{+/-}$ (*b*) mice. Photomicrographs were taken on a Zeiss IM35 inverted microscope. The same magnification was used for both micrographs. Arrowheads indicate the position of the ZP. Bar equals *ca*. 50 μm.

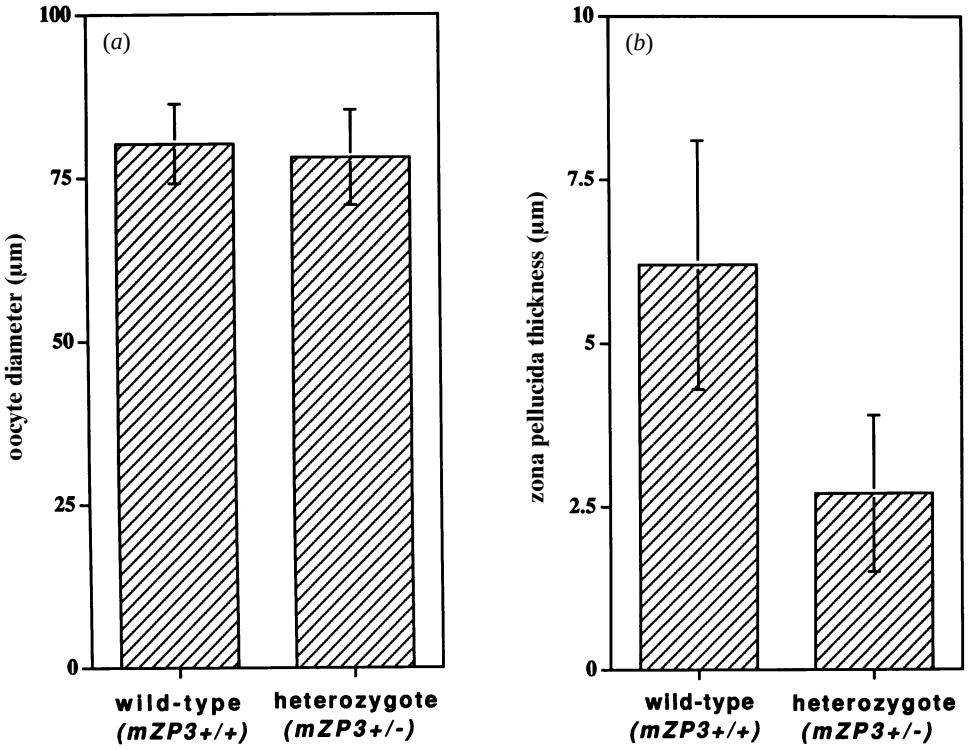

Figure 3. Estimation of the diameter of oocytes and the thickness of the ZP of oocytes recovered from ovaries of heterozygous mutant and wild-type female mice. Fully grown oocytes were recovered and examined as described in §2. (*a*) Shown is the diameter of fully grown oocytes (μm; ± s.d.) recovered 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. In each case, a minimum of 50 oocytes were measured.

(*b*) *Dimensions of ZP from* $\mathbf{mZP3^{+/-}}$ *and* $\mathbf{mZP3^{+/+}}$ *mice*

Fully grown oocytes were obtained from sexually mature mice (> four weeks of age) and eggs were obtained from superovulated females; both were examined by light microscopy, as described in §2. A picture of eggs from $mZP3^{+/-}$ and wild-type mice is shown in figure 2. Oocytes from $mZP3^{+/-}$ and $mZP3^{+/+}$ mice are approximately the same diameter (*ca*. 80 μm, excluding the ZP; figure 3) and have a ZP. However, the ZP surrounding $mZP3^{+/+}$ oocytes (*ca*. 6.2 μm thick;

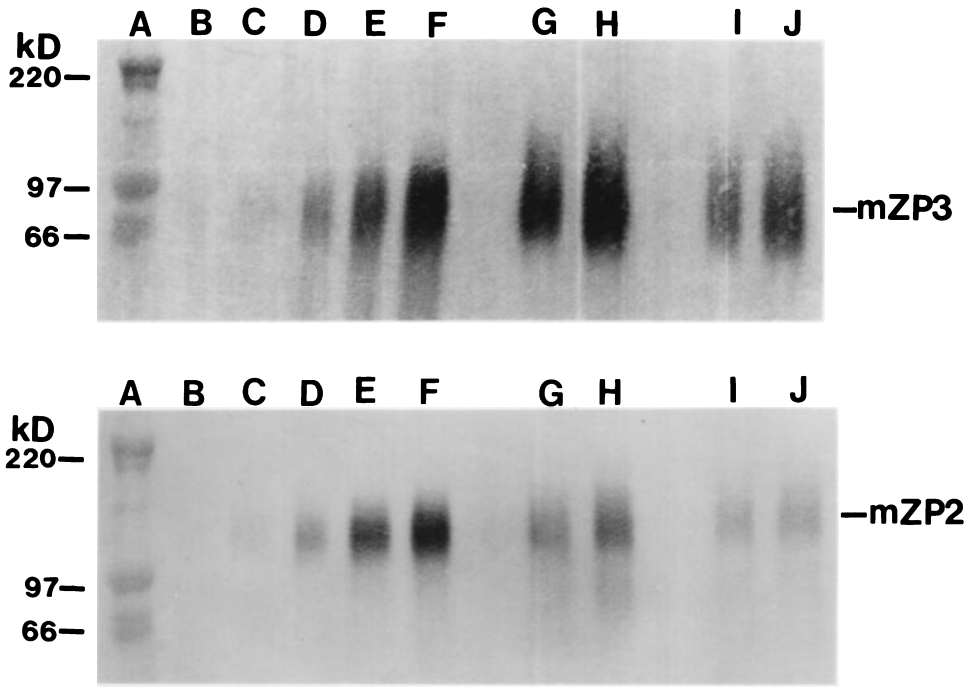

Figure 4. Estimation of the amounts of mZP3 and mZP2 in the ZP of oocytes from heterozygous mutant female mice relative to the amounts in the ZP of oocytes from wild-type female mice. Shown are Western immunoblots for mZP3 (top) and mZP2 (bottom) performed as described in §2. (Top) Lanes A–J contain: molecular weight markers (A); *ca.* 50, 100, 200, 400 and 600 ng mZP3 (B–F, respectively); mZP3 from *ca.* 1 and *ca.* 2 ovaries of wild-type mice (G and H, repectively); mZP3 from *ca.* 1 and *ca.* 2 ovaries of heterozygous mutant mice (I and J, respectively). (Bottom) Lanes A–J contain: molecular weight markers (A); *ca.* 50, 100, 200, 400 and 600 ng mZP2 (B–F, respectively); mZP2 from *ca.* 0.5 and *ca.* 1 ovaries of wild-type mice (G and H, respectively); mZP2 from *ca.* 0.5 and *ca.* 1 ovaries of heterozygous mutant mice (I and J, respectively). In each case, the gels were immunostained with antibodies directed specifically against either mZP3 or mZP2 and subjected to microdensitometry, as described in §2. Based on the microdensitometry, the ratio of mZP3 content of the ZP of heterozygous mutant as compared to wild-type mice was, on average, 0.55 ± 0.15 ($n = 4$; range 0.43–0.77); the ratio of mZP2 content of the ZP of heterozygous mutant as compared to wild-type mice was, on average, 0.44 ± 0.08 ($n = 4$; range 0.37–0.55). The molecular weight markers are 220 kDa, 97 kDa and 66 kDa M_r. The average apparent molecular weights of mZP3 and mZP2 are 83 kDa and 120 kDa M_r, respectively.

ca. 145 pl) is more than twice the width of the ZP of *mZP3*$^{+/-}$ oocytes (*ca.* 2.7 μm thick; *ca.* 56 pl; figure 3). Therefore, oocytes and eggs from mice possessing a single *mZP3* allele are about the same size as oocytes and eggs from wild-type mice, but they have a substantially thinner ZP.

(*c*) *Quantitation of mZP3 and mZP2 in ZP from* mZP3$^{+/-}$ *and* mZP3$^{+/+}$ *mice*

Since the ZP of oocytes from *mZP3*$^{+/-}$ mice were considerably thinner than the ZP of oocytes from wild-type mice (figure 3), we carried out an analysis of the relative amounts of mZP3 and mZP2 present in the ZP. Ovaries were excised from sexually mature female mice, ZP were isolated following gradient centrifugation of ovarian homogenates, and mZP3 and mZP2 were quantitated following SDS/PAGE and Western immunoblotting of isolated ZP, as described in §2. As seen in figure 4, the ZP of oocytes from *mZP3*$^{+/-}$ mice contained significantly less mZP3 and mZP2 than the ZP of oocytes from wild-type mice. Quantitation of these results revealed that the ZP from heterozygous mutant mice contained, on average, *ca.* 44% ($\pm$ 8%) of the mZP2 and *ca.* 55% ($\pm$15%) of the mZP3 present in ZP from wild-type mice (figure 4). These results are consistent with the observed differences in ZP thickness (figure 3) and calculated differences in ZP volumes.

(*d*) *Reproduction of* mZP3$^{+/-}$ *mice*

Despite the presence of a thinner than usual ZP surrounding their eggs, *mZP3*$^{+/-}$ females reproduced normally. 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 (average of eight litters); in matings of *mZP3*$^{-/-}$ males with *mZP3*$^{+/+}$ females, the average litter contained 7.8 pups (average of eight 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.

4. DISCUSSION

During each reproductive cycle, as ovarian mouse oocytes grow, a ZP is deposited around the entire oocyte (Wassarman 1996). The ZP increases in

thickness as the oocyte increases in diameter over a 2–3-week period of oogenesis. The ovulated mouse egg (*ca.* 75 μm in diameter) has a ZP that is approximately 6.5 μm thick. It should be noted that, although all mammalian eggs are surrounded by a ZP, the ZP of eggs from different mammalian species varies considerably in thickness, from < 2 μm to > 25 μm (Dunbar *et al.* 1991).

The ZP of mouse oocytes is composed of three glycoproteins, mZP1–3, which are synthesized and secreted by the growing oocyte during the latter stages of oogenesis, just preceding ovulation (Wassarman *et al.* 1985; Kinloch *et al.* 1993; Epifano & Dean 1995). mZP3 (*ca.* 83 kDa M_r) and mZP2 (*ca.* 120 kDa M_r) are the major constituents of the ZP, forming the long, interconnected filaments that are the structural basis of the ZP (Greve & Wassarman 1985; Wassarman 1988; Wassarman & Mortillo 1991). mZP3 and mZP2 are present in the ZP in approximately equimolar amounts and interact with each other by using non-covalent bonds (Wassarman 1988). mZP1 (*ca.* 200 kDa M_r) is present in relatively low amounts in the ZP, as compared to mZP3 and mZP2, and apparently serves as the crosslinker that interconnects ZP filaments (Greve & Wassarman 1985; Wassarman & Mortillo 1991).

It is clear from the results of two types of recent experiments that mZP3 and mZP2 must be incorporated simultaneously into the nascent ZP. In one set of experiments, a large excess of complementary oligonucleotide was injected into the cytoplasm of isolated growing mouse oocytes in order to target degradation of either mZP2 or mZP3 messenger RNA (Tong *et al.* 1995). Within 16 h of injection, the targeted ZP glycoprotein was no longer synthesized by the oocyte, whereas the non-targeted glycoprotein continued to be synthesized. Under these conditions, it was found that the absence of synthesis of either glycoprotein prevented incorporation of the other glycoprotein into the ZP. In a different set of experiments, mZP3 null mutant mice ($mZP3^{-/-}$) were produced using homologous recombination in ES cells and standard gene targeting procedures (Liu *et al.* 1995, 1996; Rankin *et al.* 1996; Wassarman *et al.* 1996). In the complete absence of mZP3 synthesis, growing and fully grown oocytes, as well as unfertilized eggs, lacked a ZP. Thus, despite the fact that mZP2 continued to be synthesized (Rankin *et al.* 1996; P. Wassarman, unpublished results), the complete elimination of mZP3 synthesis prevented assembly of a ZP during oogenesis.

Here, we examined the ZP of oocytes from female $mZP3^{+/-}$ mice that are fertile (Liu *et al.* 1995, 1996; Wassarman *et al.* 1996). Oocytes from these mice have a ZP, but it is considerably thinner than the ZP surrounding oocytes from wild-type mice (figures 2 and 3). Despite this, the mice reproduce normally. The lack of an effect of a relatively thin ZP on fertilization of mouse eggs is of interest in connection with certain other observations. For example, previously it was found that sperm bound very well to the ZP of growing mouse oocytes at several stages of the growth phase (Bleil & Wassarman 1980). The ZP surrounding these oocytes was as little as half the thickness of the ZP of fully grown oocytes and unfertilized eggs. This suggests that, even with a relatively thin ZP, a sufficient number of copies of mZP3 are available to support binding of free-swimming sperm.

In view of the altered width of the $mZP3^{+/-}$ ZP, we have estimated the relative amounts of mZP3 and mZP2 present in the ZP of oocytes from $mZP3^{+/-}$ mice, as compared to $mZP3^{+/+}$ mice. The data suggest that, indeed, there are reduced levels of both mZP3 and mZP2 in ZP of oocytes from $mZP3^{+/-}$ mice (figure 4). The amount of mZP3 present (*ca.* 55 % of wild-type; *ca.* 0.9 ± 0.25 ng/ZP, assuming that the wild-type mouse ZP contains *ca.* 3.5 ng protein) is consistent with the presence of one, rather than two *mZP3* alleles, and with the absence of dosage compensation. The amount of mZP2 present in the ZP (*ca.* 44 % of wild-type; *ca.* 0.7 ± 0.12 ng/ZP, assuming that the wild-type mouse ZP contains *ca.* 3.5 ng protein) is consistent with current models for ZP structure in which ZP filaments are constructed of mZP2-mZP3 dimers (Wassarman & Mortillo 1991; Wassarman 1996), and with the presence of limiting amounts of mZP3. That is, in the presence of approximately half the wild-type amount of mZP3, only 40–50 % of nascent mZP2 can be accommodated in ZP filaments. When no mZP3 is present (e.g. in $mZP3^{-/-}$ mice), nascent mZP2 cannot be assembled into a ZP and, as a result, oocytes and eggs lack a ZP and the females are infertile. Whether or not unfertilized eggs from $mZP3^{-/-}$ mice can be fertilized *in vitro*, and give rise to viable offspring when transplanted into pseudopregnant foster mothers, remains to be determined.

In conclusion, the results of these experiments with $mZP3^{+/-}$ mice strongly support the proposed molecular model of the ZP (Wassarman 1988, 1996). As mZP2, like mZP3, is a structural component of the ZP, it is to be expected that targeted disruption of the *mZP2* gene would have similar effects on ZP assembly in homozygous and heterozygous female mice. It will be of interest to examine the intracellular pathway of assembly of the ZP in growing oocytes in much more detail in order to account for certain features of these mutant mice.

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