JOURNAL OF EXPERIMENTAL ZOOLOGY (MOL DEV EVOL) 285:251–258 (1999)

Structure and Function of the Mammalian Egg Zona Pellucida

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ABSTRACT The zona pellucida is a thick extracellular coat that surrounds all mammalian eggs and preimplantation embryos. The zona pellucida supports communication between oocytes and follicle cells during oogenesis; protects oocytes, eggs, and embryos during development, and regulates interactions between ovulated eggs and free-swimming sperm during and following fertilization. Mutant females that produce eggs that lack a zona pellucida are infertile. The functions of the zona pellucida during fertilization now can be ascribed to certain of its glycoproteins. Here we describe some aspects of zona pellucida structure and function as they relate to mammalian fertilization. *J. Exp. Zool. (Mol. Dev. Evol.) 285:251–258, 1999.* © 1999 Wiley-Liss, Inc.

There is general agreement that the zona pellucida (ZP) serves as a barrier (admittedly, not always an effective barrier) to fertilization of eggs from one species by sperm from another species (Gwatkin, '77; Yanagimachi, '94; Wassarman, '99) (Fig. 1). Traditionally, it has been thought that the egg ZP contains species-specific sperm receptors that can be recognized primarily by sperm from the same species. Furthermore, the ZP serves as a barrier to polyspermic fertilization. Once an egg has fused with a single sperm to form a zygote, free-swimming sperm are excluded from binding to the ZP. This has been attributed to an inactivation of sperm receptors in the ZP shortly after fertilization.

During the past two decades, some of the conjecture associated with the ZP has been re-examined experimentally. Perhaps, most revealing, was the finding that the mouse egg ZP consists of only three different glycosylated proteins (Bleil and Wassarman, '80a). Each of these glycoproteins consists of a unique polypeptide that is heterogeneously glycosylated. Therefore, the functions of the ZP during fertilization must be carried out by a very small number of glycoprotein components.

ZONA PELLUCIDA GLYCOPROTEINS

The mouse egg ZP (~6.2 μm thick containing ~3.5 ng of protein) is composed of three glycoproteins, called mZP1 (~200 kDa; dimer of identical polypeptides), mZP2 (~120 kDa), and mZP3 (~83 kDa) (Bleil and Wassarman, '80a; Wassarman, '88, '99). Each of these glycoproteins consists of a unique polypeptide (mZP1, 2-times ~75 kDa; mZP2, ~75 kDa; mZP3, ~44 kDa) that is heterogeneously glycosylated with both complex-type aspargine- (N-) linked and serine/threonine- (O-) linked oligosaccharides. As a consequence of the glycosylation, each of the glycoproteins is relatively acidic and appears as a broad band following gel-electrophoresis. Each of the polypeptides possesses domains that are conserved among ZP glycoproteins (e.g., signal sequences, consensus furin cleavage-sites, hinge-regions, hydrophobic regions, and so-called ZP domains) (Fig. 2). For example, it is now apparent that nascent mZP2 and mZP3 polypeptides are cleaved by proteases both at their N-terminus (signal sequence) and their C-terminus (consensus furin cleavage-site) prior to incorporation into the ZP (E. Litscher, H. Qi, and P. Wassarman, unpublished results).

Mouse ZP glycoproteins are synthesized and secreted by growing oocytes (Bleil and Wassarman, '80b). Genes encoding polypeptides of mouse ZP glycoproteins are expressed exclusively by growing oocytes and are regulated by *cis*-acting sequences located close to the transcription start-site and by several *trans*-acting

Grant sponsor: National Institute of Child Health and Human Development; Grant number: HD-35105.
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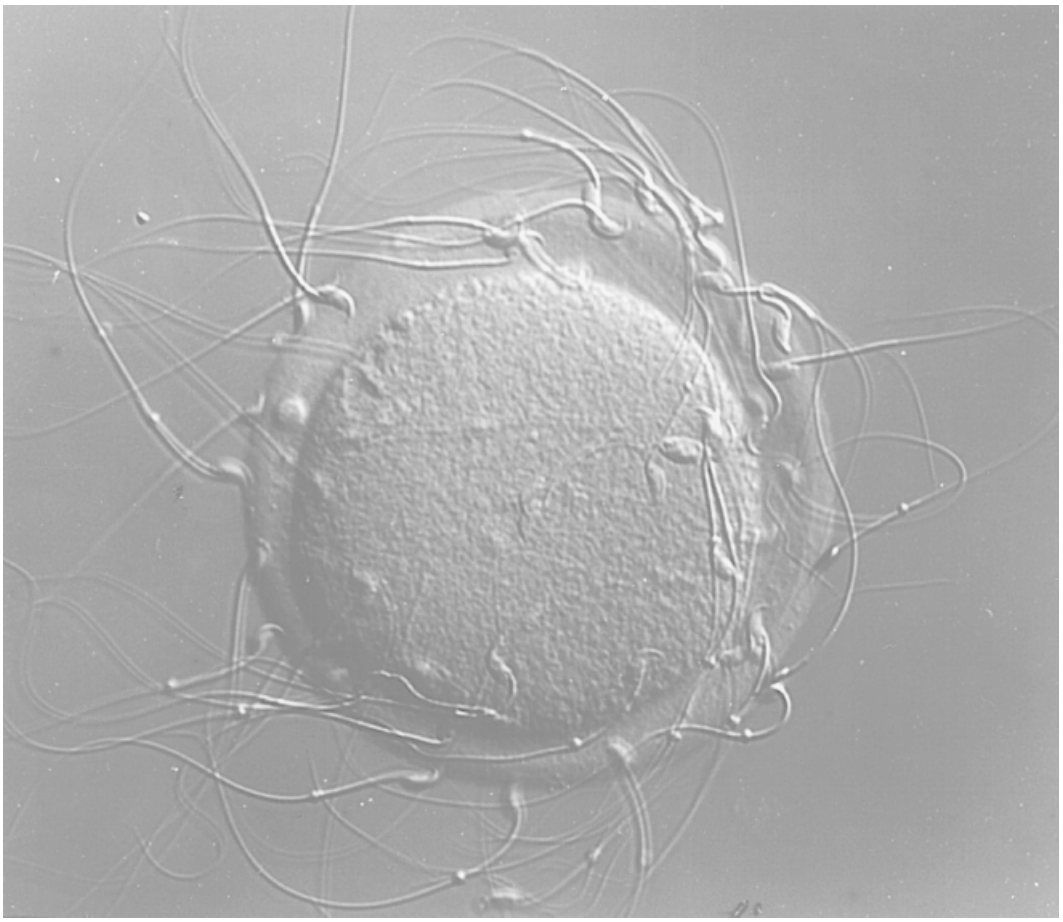

Fig. 1. Photomicrograph (Nomarski DIC) of mouse sperm bound to the zona pellucida of an unfertilized mouse egg in vitro (×~800).

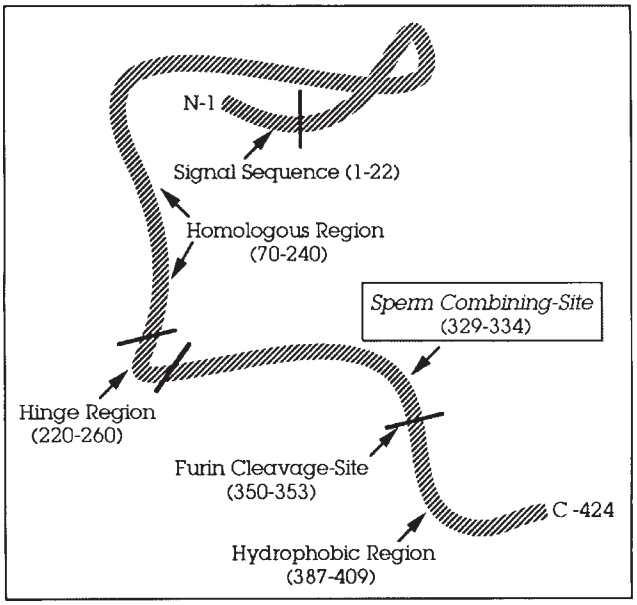

Fig. 2. Schematic diagram of the mZP3 polypeptide with specific structural and functional domains indicated. It should be noted that the N-terminal signal sequence (amino acids 1–22) and C-terminal fragment (originating at the consensus furin cleavage-site and encoded by exon-8) are missing from mZP3 present in the ZP.

factors (Lira et al., '90, '93; Schickler et al., '92; Kinloch et al., '93; Millar et al., '93; Liang et al., '97). Although there remains some debate about the site of synthesis of ZP glycoproteins in some species other than mice (i.e., oocytes and/or follicle cells), this issue should be resolved shortly.

Today, it is clear that the ZP of eggs from a wide variety of mammals, including humans, is composed of a small number of glycoproteins that are closely related (polypeptides ~40–90% similar) to mZP1–mZP3. For example, the positions of the cysteine residues, as well as several of the recognizable domains of mZP3 polypeptide, are conserved in human ZP3 polypeptide. Even the vitelline envelope (VE) surrounding eggs from fish, birds, and amphib-

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ians contains glycoproteins whose polypeptides resemble mZP1-mZP3. Thus, there is an evolutionary link between glycoproteins of the VE of nonmammalian eggs and glycoproteins of the ZP of mammalian eggs. Since these glycoproteins play essential structural roles in assembling the extracellular coats during oogenesis (although their synthesis takes place in different sites; e.g., in growing oocytes, follicle cells, or liver), it is not surprising that they retain common features. It will be of great interest to determine and compare the high-resolution structures of ZP glycoproteins and their VE counterparts.

ZONA PELLUCIDA STRUCTURE

It has been proposed that the three mouse ZP glycoproteins are organized in a very specific manner. In this model, the ZP is composed of long interconnected filaments that are polymers of mZP2 and mZP3 (Greve and Wassarman, '85; Wassarman and Mortillo, '91). An mZP2–mZP3 dimer is located every 140 Å or so along the filaments, thus imposing a structural periodicity that can be seen in electron micrographs of dissolved ZP. The filaments, in turn, are cross-linked by mZP1 to create a three-dimensional matrix. We recently suggested that ZP assembly from nascent glycoproteins is a stochastic process that may occur completely outside the growing oocyte itself (Qi and Wassarman, '99).

Support for this view of ZP structure has come from two different experimental approaches:

1. Using an antisense approach, injection of a large excess of complementary oligonucleotide into the cytoplasm of isolated mouse oocytes can target degradation of either mZP2 or mZP3 messenger-RNA (Tong et al., '95). Within 16 hr 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.
2. Using a genetic approach, the *mZP3* gene was disrupted by targeted mutagenesis using homologous recombination in mouse embryonic stem (ES) cells. Homozygous (Liu et al., '96; Rankin et al., '96; Qi and Wassarman, '99) and heterozygous (Wassarman et al., '97) *mZP3* null mutant females were produced and, in both cases, obvious effects were observed on ZP assembly. In the presence of mZP1 and mZP2 synthesis, but the complete absence of mZP3 synthesis *(mZP3*$^{-/-}$ mice), growing oocytes and ovulated eggs lacked a ZP and the females were infertile (Fig. 3). On the other hand, when lower than wild-type amounts of mZP3 were synthesized by heterozygous *mZP3* null mice *(mZP3*$^{+/-}$ mice), growing oocytes and ovulated eggs had a relatively thin ZP (approximately one-half the thickness of the wild-type ZP), but the females were fertile

Results of these antisense and "knockout" experiments are very revealing with respect to ZP assembly. They strongly suggest that the presence of both mZP2 and mZP3 (1:1 equimolar) absolutely is required for assembly of a wild-type ZP. For example, when nascent mZP3 is completely missing *(mZP3*$^{-/-}$*)*, no ZP is assembled, and only low levels of nascent mZP3 are present *(mZP3*$^{+/-}$*)*, a relatively thin ZP is assembled. These observations are consistent with the model in which mZP2 and mZP3 are present in ZP filaments as heterodimers. Modulation of the level of one nascent ZP glycoprotein affects incorporation of the other glycoprotein into the ZP.

It is of interest to note that *mZP3*$^{-/-}$ female mice also exhibit retarded follicular development as compared to wild-type animals. This difference is reflected in the size of ovaries recovered from juvenile and adult mice (Fig. 4). In general, ovaries from *mZP3*$^{-/-}$ mice contain few, if any, Graafian follicles and it is apparent that the failure to produce a ZP around growing oocytes has pleiotropic effects on follicle development.

mZP3 IS A SPERM RECEPTOR

Only acrosome-intact sperm bind to the ovulated mouse egg ZP. Experimental evidence strongly supports the conclusion that during binding of sperm to eggs, mZP3 serves as a receptor for sperm. For example, of the three glycoproteins that constitute the ZP, only purified mZP3 binds exclusively to heads of acrosome-intact sperm, thereby preventing sperm from binding to ovulated eggs in vitro (Bleil and Wassarman, '80c, '86; Wassarman, '90; Mortillo and Wassarman, '91). Even at nanomolar concentrations, purified, unfertilized egg mZP3 is a very effective inhibitor of sperm binding in this competition assay. On the other hand, at similar concentrations, mZP3 from fertilized eggs or early embryos has no effect on binding of sperm to eggs in vitro. This is consis-

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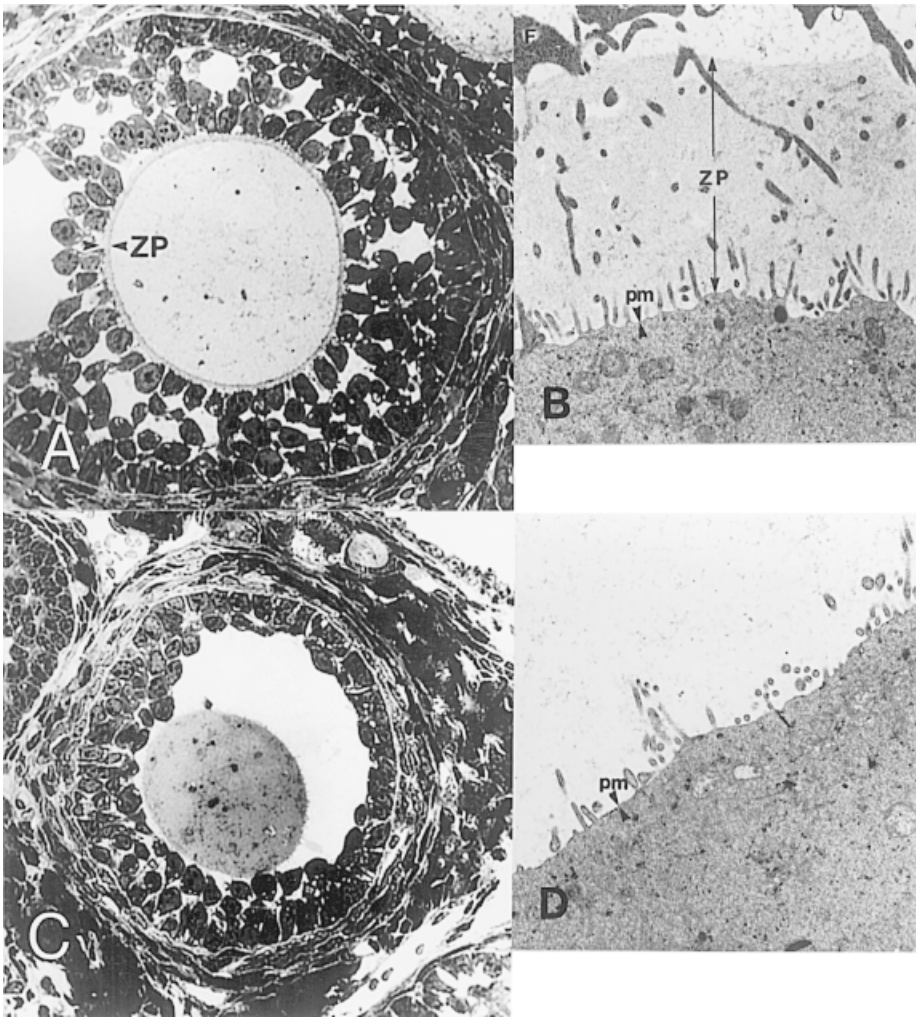

Fig. 3. Light and electron micrographs of ovarian follicles and growing oocytes in wild-type and *mZP3*$^{-/-}$ mice. (**A** and **C**) Light micrographs of ovarian follicles in ~6-week-old wild-type (A) and *mZP3*$^{-/-}$ (C) mice. Note the ZP and follicle cells in close apposition to the growing oocyte in the follicle from the wild-type mouse (A). Note the complete lack of a ZP and the lack of intimate contact between the oocyte and follicle cells in the follicle from the *mZP3*$^{-/-}$ mouse (C). (**B** and **D**) Electron micrographs of growing oocytes, ~65–70 μm in diameter, isolated from 17-day-old wild-type (B) and *mZP3*$^{-/-}$ (D) mice. Note the thick ZP around the wild-type oocyte (B) that is completely missing from the *mZP3*$^{-/-}$ oocyte (D). zp, zona pellucida; f, follicle cell; pm, plasma membrane. (A and C, ×~230; B and C, ×~5,000.)

tent with the failure of free-swimming sperm to bind to the ZP of fertilized eggs and preimplantation embryos. It can be concluded from these and other observations that, as a consequence of the zona reaction following fertilization, mZP3 is altered such that free-swimming sperm no longer can recognize and bind to the glycoprotein.

mZP3 IS AN ACROSOME REACTION-INDUCER

The acrosome is a large secretory vesicle that overlies the nucleus in the apical region of the sperm head (Yanagimachi, '94; Wassarman, '99). Acrosomal membrane just underlying the plasma membrane is referred to as "outer" acrosomal membrane and that overlying the nucleus is referred to as "inner" acrosomal membrane. Morphologically, the AR is seen as multiple fusions between the outer acrosomal membrane and the plasma membrane at the anterior region of sperm head, extensive formation of hybrid membrane vesicles, and exposure of the inner acrosomal membrane and acrosomal contents. Only acrosome-reacted sperm can penetrate the ZP and fuse with egg plasma membrane.

It is known that there are many different inducers of the AR (Yanagimachi, '94). However, it is now generally accepted that ZP3 is the natural agonist that initiates the AR upon binding of acrosome-

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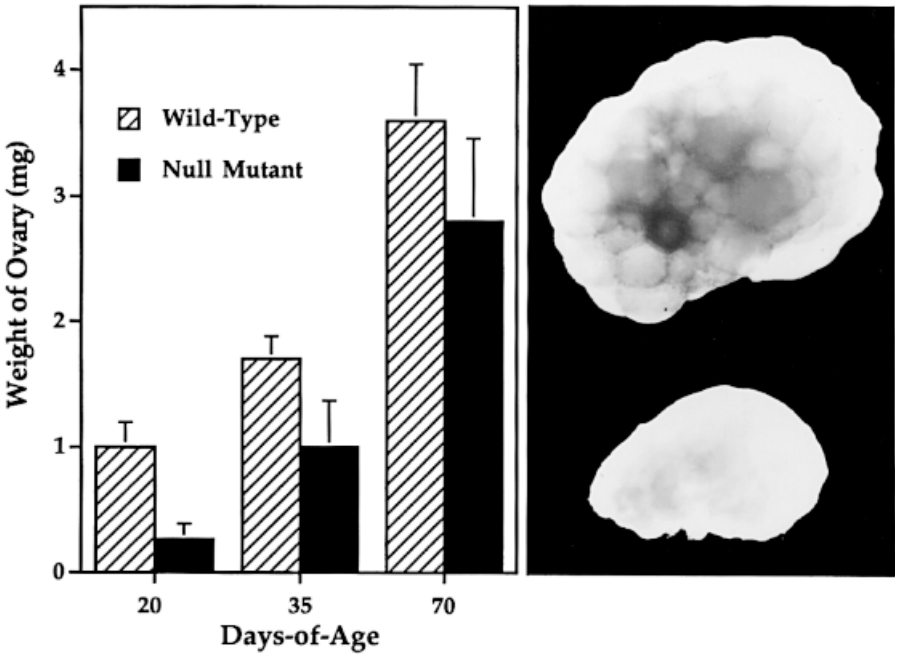

Fig. 4. Estimates of the weights of ovaries excised from wild-type and $mZP3^{-/-}$ mice. Shown are average weights (mean ± sd; mean of 16–22 ovaries) of ovaries excised from wild-type (hatched bars) and mutant (solid black bars) mice as a function of age. Also shown is a photomicrograph of ovaries excised from a 20-day-old wild-type (top) and a 20-day-old mutant (bottom) mouse. Note the large number of antral follicles ready to be ovulated on the surface of the ovary excised from a wild-type mouse. Few, if any, antral follicles are present on the surface of the ovary excised from a $mZP3^{-/-}$ mouse (×~25).

intact sperm to the ZP (Bleil and Wassarman, '83; Darszon et al., '96; Florman et al., '98). Binding of sperm to ZP3 activates sperm G proteins and voltage-sensitive Ca^{2+} channels (T-type channels) and results in a depolarization of the sperm membrane and a significant increase (2–3-fold) in intracellular Ca^{2+} levels. Criteria are available that permit one to distinguish between the so-called "spontaneous" AR and the ZP3-induced AR (e.g., sensitivity to pertussis toxin). While purified mZP3 and large mZP3 glycopeptides induce sperm to acrosome-react in vitro, small mZP3 glycopeptides and purified mZP3 O-linked oligosaccharides bind to sperm and inhibit their binding to eggs, but do not induce the AR (Wassarman, '88, '90). In the latter context, it has been reported that cross-linking of small mZP3 glycopeptides bound to sperm can induce the sperm to acrosome-react (Leyton and Saling, '89). These and other findings suggest that induction of the AR by ZP3 will turn out to be dependent on multivalent interactions.

SPERM BIND TO SPECIFIC mZP3 OLIGOSACCHARIDES

Interestingly, the ability of mZP3 to act as a sperm receptor in vitro is not significantly affected by high temperatures, detergents, denaturants, reducing agents, or limited proteolysis. Even after extensive proteolysis of mZP3, the small glycopeptides produced retain sperm receptor activity, although higher than normal concentrations of glycopeptides are required (Florman et al., '83; Florman and Wassarman, '85; Litscher and Wassarman, '96). These and other observations suggest that mZP3 polypeptide does not play a direct role in sperm receptor function.

However, there is considerable evidence to suggest that mZP3 oligosaccharides do play a direct role in sperm receptor function. For example, chemical removal of all mZP3 oligosaccharides results in complete inactivation of the glycoprotein as a sperm receptor. Furthermore, O-linked oligosaccharides recovered from mZP3 by mild alkaline hydrolysis under reducing conditions (Florman and Wassarman, '85) and certain O-linked related oligosaccharides (Litscher et al., '95) inhibit binding of sperm to eggs in vitro at micromolar concentrations. Collectively, these and other observations suggest that species-specific binding of sperm to eggs in mammals is a carbohydrate-mediated event. On the other hand, the identity of sugars on mZP3 oligosaccharides rec-

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ognized by sperm remains an unresolved and controversial issue.

LOCATION OF ESSENTIAL mZP3 OLIGOSACCHARIDES

To locate essential O-linked oligosaccharides on mZP3 polypeptide, we used limited proteolysis (Rosiere and Wassarman, '92; Litscher and Wassarman, '96), exon swapping (Kinloch et al., '95), and site-directed mutagenesis (Kinloch et al., '95; Chen et al., '98). Results of such studies suggest that the essential oligosaccharides are present on just two of five Ser residues, Ser-332 and -334, in a region of polypeptide near the C-terminus; a region encoded by exon-7 of the *mZP3* gene (8 exons) (Fig. 5). Interestingly, of the five Ser residues, only these two are conserved from mouse to human ZP3. In this context, the numerous amino acid changes neighboring Ser-332 and -334 that have occurred during evolution may impose changes in the structure of O-linked oligosaccharides added to ZP3 and, thereby, affect species specificity of sperm-egg interaction (Wassarman and Litscher, '95) (Fig. 6).

CONCLUSION

In this brief review we tried to highlight some of the important features of mammalian egg ZP structure and function. Some of the points made are: (1) The ZP is composed of only three glycoproteins, mZP1-3, that are evolutionarily conserved; (2) two of the ZP glycoproteins, mZP2 and mZP3, are organized into long filaments exhibiting a periodicity and the third glycoprotein, mZP1, serves as a cross-linker of filaments; (3) the presence of both mZP2 and mZP3 is absolutely required for assembly of the ZP during oocyte growth; (4) female mice are infertile when their eggs lack a ZP; (5) mZP3 serves as the primary sperm receptor and inducer of the acrosome reaction during fertilization; (6) binding of sperm to egg mZP3 is a carbohydrate-mediated event attributable, in part, to specific O-linked oligosaccharides on mZP3; (7) the mZP3 oligosaccharides recognized by sperm are located on two conserved Ser residues encoded by exon-7 of the *mZP3* gene.

Although we have learned a great deal about mZP3 and its roles in fertilization, many other issues remain to be resolved. For example, the nature of the protein component associated with plasma membrane surrounding the sperm head that recognizes and binds to mZP3 oligosaccharides (so-called egg-binding protein or EBP) is not known. This area of research, while extremely productive, to date is inconclusive (Wassarman, '99). The nature of the ZP3 oligosaccharides that are recognized by EBPs also must be determined. We know very little about the structure of these oligosaccharides (e.g., composition, sequence, and conformation) or their sites on ZP3 polypeptides from different mammalian eggs. Do these oligosaccharides account for the species-specific binding of sperm to eggs? Finally, it remains for us to work out the mechanism for inactivation of mZP3 as a sperm receptor following fertilization. Although it appears to involve alteration of mZP3 O-linked

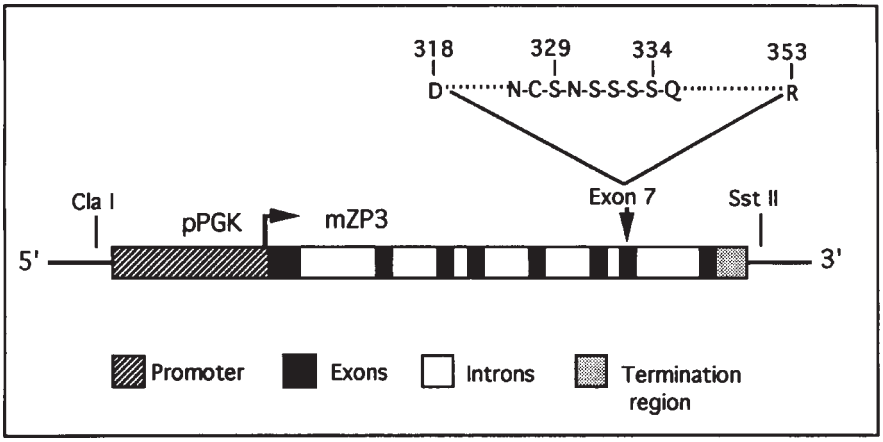

Fig. 5. Schematic diagram of the *PGK/mZP3* recombinant gene used to generate stably transfected EC-mZP3 cell lines. *pPGK-1* represents the mouse phosphoglycerate kinase-1 promoter region. Restriction enzymes *Cla*I and *Sst*II were used to generate linearized DNA fragments for electroporation of EC (F9) cells. Arrow indicates the transcriptional start-site on the *PGK-1* promoter. The sites of mutagenesis in exon-7 of the *mZP3* gene are indicated as amino acids 329–334. In each case, the amino acid was changed to Ala, Val, or Gly (i.e., a non-hydroxyl amino acid).

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M   MASSYFLFLCLLLCGGPELCNS QTLWLLPGGTPTPVGSSS 40
H   .GL..Q.L........AKQ.C. .P...........GKLT. 40
U   .EL..R..I....W.ST...YP  .P....Q..ASH.ET.VQ 40

M   PVKVECLEAELVVTVSRDLFGTGKLVQPGDLTLGSEGCQP  80
H   S.E..._..................I..E.......N_R.  80
U   ....._Q..T.M.M..K........IRAA.....P.A_E.  80

M   RVSVDTDVVRFNAQLHECSSRVQMTKDALVYSTFLLHDPR  120
H   L...A......K....._.N...V.E.......V...Q..  120
U   L..M.......EVG..._GNSM.V.D..............  121
        +E

M   PVSGLSILRTNRVEVPIECRYPRQGNVSSHPIEPTWVPFR  160
H   ..P.........AD...._......._....A.R......S  160
U   ..GN...V....A.I..._......._...QA.L...L...  161

M   ATVSSEEKLAFSLRLMEENWNTEKSAPTFHLGEVAHLQAE  200
H   T........V..............LS.........Y....  200
U   T..F.....T...........A..RS......DA......  201

M   VQTGSHLPLQLFVDHCVATPSPLPDPNSSPYHFIVDFHGC  240
H   .........L....R_......--.QTA....V......._  238
U   IH....V..R....._...--.T..Q_A....T......._  239

M   LVDGLSESFSAFEVPRPRPETLQFTVDVFHFANSSRNTLY  280
H   ............Q....................._.....I.  278
U   .....TDAS...K....G.D............_D...MI.  279

M   ITCHLKVAPANQIPDKLNKACSFNKTSQSWLPVEGDADIC  320
H   .._....T.._.T..E...._.._RS.K..S......EV_  318
U   .._....TL.E.D..E...._..S.P.N..F....P..._  319

M   DCCSHGNCSNSSSSQFQIHGPRQWSKLVSRNRRHVTDEAD  360
H   G_._.S.D_GS..R.RY.A..VS..P.SA..R....R....  358
U   Q_._NK.D_GTP.H.RR.P.VMS...ASA........E...  359

M   VTVGPLIFLGKANDQTVEGWTASAQTSVALGLGLATVAFL  400
H   ............S..A....AS.....L.......A....  398
U   .........DA.G.HE..Q.ALPSD...L..V...V.VS.  400
                                     +V

M   TLAAIVLAVTRKCHSSSYLVSLPQ  424
H   .......G...S_.TP.HV...S.  422
U   ..T.VI.VL..R_RTA.HP..ASE  424
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Fig. 6. Comparison of the primary structures of mouse (M), hamster (H), and human (U) ZP3 polypeptides using the single-letter amino acid code. For hamster and human ZP3, only those positions at which the amino acid sequence differs from that of mouse ZP3 are shown. A two amino acid deletion in hamster ZP3 is indicated by dashes at positions 223 and 224 of mouse ZP3. A two amino acid deletion in human ZP3 is indicated by dashes at positions 220 and 221 of mouse ZP3. Two added amino acids, E and V in human ZP3, are indicated just below the sequence. The predicted signal sequences are italicized (residues 1–22). Locations of 13 cysteine residues (C) and potential N-linked glycosylation sites (N) are underlined. The mouse consensus furin cleavage-site, -R-N-R-R-, is located at residues 350–353.

oligosaccharides by cortical granule glycosidases following fertilization, further experimentation is necessary. We hope that answers to these and other questions will be forthcoming and that such progress will be of some benefit to clinicians and their patients involved in the manipulation of human reproduction.

ACKNOWLEDGMENTS

We especially thank Dr. Maurizio Zuccotti for his kind invitation to participate in the 1999 Lazzaro Spallanzani Symposium in Pavia.

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