The Parkes Lecture*

Zona pellucida glycoprotein mZP3: a versatile player during mammalian fertilization

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All mammalian eggs are surrounded by a relatively thick extracellular coat, the zona pellucida (ZP), which facilitates fertilization of eggs by a single spermatozoon. The mouse egg ZP is constructed of only three glycoproteins, termed mZP1–3. Each of these glycoproteins consists of a unique polypeptide that is heterogeneously glycosylated with both asparagine-(N)-linked and serine/threonine-(O)-linked oligosaccharides. Polypeptides of ZP glycoproteins are highly conserved among mammalian species and are similar to polypeptides of egg vitelline envelope glycoproteins of fish, birds and amphibians. One of the mouse ZP glycoproteins, mZP3, serves as both a receptor for spermatozoa and an inducer of the acrosome reaction during fertilization. Free-swimming acrosome-intact spermatozoa recognize and bind to certain serine-(O)-linked oligosaccharides located close to the carboxy terminus of mZP3 polypeptide and, after binding, undergo the acrosome reaction (cellular exocytosis). In this review, in addition to the background information presented, results of recent experiments using homologous recombination to produce mZP3 null mice and site-directed mutagenesis to inactivate mZP3 as a sperm receptor and inducer of the acrosome reaction are presented and discussed.

Introduction

For more than two decades, my colleagues and I have carried out research on a single aspect of mammalian fertilization, the species-specific binding of free-swimming spermatozoa to ovulated eggs (Fig. 1). In particular, our research has focused on the identification and characterization of molecules that support this initial step in the fertilization pathway in mice. In this review, I describe one such molecule, a zona pellucida glycoprotein called mZP3, which performs multiple functions during mammalian fertilization. This glycoprotein is unique to mammalian eggs, from mice to humans, although its ancestors can be found in vitelline envelopes from a wide variety of vertebrate eggs.

Mammalian oogenesis

Oogenesis in mammals begins early in fetal development and ends, months to years later, in the sexually mature adult (Wassarman and Albertini, 1994). Oogenesis begins with formation of primordial germ cells (PGC) and proceeds through a series of cellular transformations, from PGC to oogonia to oocytes in the fetus, and from oocytes to eggs in

the adult. Oogonia proliferate by mitosis, whereas oocytes are meiotic cells.

In mice, the germ cell line has extragonadal origins and originates before or during primitive streak formation in embryos of approximately 7 days of age. At approximately 13 days of age, embryos contain a differentiated ovary and migration of PGC is complete; virtually all of the cells have converted to dividing oogonia in the sex cords. As early as day 12 of embryogenesis, a few oogonia enter the preleptotene and then the leptotene stage of the first meiotic prophase; during preleptotene the final round of DNA replication occurs and oogonia become oocytes. It takes approximately 4 days to complete nuclear progression from leptotene to pachytene. By parturition at approximately day 21, most oocytes have entered late diplotene or the dictyate stage, and by day 5 post partum, nearly all oocytes have reached the dictyate stage, where they remain until they are stimulated to resume meiosis at the time of ovulation. This large pool of small (12–15 μm in diameter) non-growing oocytes is the sole source of unfertilized eggs in the sexually mature adult mammal.

In adult mammals, growth of oocytes is continuous, and ends in either ovulation of a matured oocyte (unfertilized egg) or degeneration of the oocyte and its follicle. Completion of oocyte growth in mice takes 2–3 weeks, during which the diameter of the oocytes increases from 12 to 80 μm , not including the zona pellucida (ZP); this represents about a 300-fold increase in volume. Just before

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Fig. 1. Photomicrograph of mouse spermatozoa bound to the zona pellucida of an unfertilized mouse egg *in vitro*.

ovulation, after the preovulatory gonadotrophin (FSH and LH) surge, fully grown oocytes undergo meiotic maturation and become unfertilized eggs arrested at metaphase II of meiosis. The ZP first appears during the earliest stages of oocyte growth, and increases in thickness continually as oocytes increase in diameter.

Mammalian fertilization

The final steps of mammalian oogenesis and spermatogenesis prepare eggs and spermatozoa, respectively, for fertilization (Gwatkin, 1977; Yanagimachi, 1994; Snell and White, 1996; Wassarman, 1987, 1999). During ovulation, fully grown oocytes from antral follicles undergo meiotic maturation and become unfertilized eggs that are ready to interact with spermatozoa. Similarly, after deposition into, and migration up, the female reproductive tract, spermatozoa undergo capacitation, a process that enables them to bind to eggs and undergo the acrosome reaction (AR). Meiotic maturation of oocytes and capacitation of spermatozoa set gametes off on a path that leads to either formation of a viable zygote or degeneration of the cells.

Typically, very few ovulated eggs are found in the oviducts of females (approximately ten in mice). Similarly, relatively few spermatozoa are found at the site of fertilization (approximately 125 in mice) compared with the number of spermatozoa deposited into the female reproductive tract (approximately 10^7). A very small percentage of ejaculated spermatozoa reach the position of unfertilized eggs in the oviduct. Whether binding of spermatozoa to eggs occurs due to a chance encounter of gametes in the oviduct or is promoted by a chemical gradient

stimulus (sperm chemotaxis), as observed in many non-mammalian species, remains to be resolved conclusively.

Among mammals, the process of the union of germ cells includes several steps that take place in a compulsory order (Fig. 2). It begins in the oviduct with binding of free-swimming spermatozoa to the ovulated egg ZP and ends a short time later (1–2 h after combining gametes *in vitro*) with fusion of egg and sperm plasma membranes to form a single activated cell, the zygote. During this process, several recognizable events take place, including the sperm AR (a form of cellular exocytosis), penetration of the egg ZP by the spermatozoon, and the egg cortical reaction and zona reaction. The latter results in alteration of the ZP such that free-swimming spermatozoa are unable to bind to fertilized eggs or preimplantation embryos (Fig. 3).

Does mammalian fertilization show any species specificity? It is well known that interspecific hybrids of certain mammals are viable. However, evidence from *in vitro* fertilization experiments indicates that there are indeed barriers to interspecific fertilization and that the egg ZP serves as a major barrier. The ZP can interfere with interspecific fertilization by failing to permit the initial binding of spermatozoa to eggs, induction of the AR, or penetration of bound spermatozoa through the extracellular coat. Although the restrictions on binding are not absolute, they provide a relatively high degree of species-specific fertilization *in vitro*. Notably, removal of the ZP from unfertilized mammalian eggs, thereby exposing egg plasma membrane directly to spermatozoa, eliminates the barrier to interspecific fertilization *in vitro* for many, but not all mammals (Yanagimachi, 1977, 1981, 1994).

Zona pellucida glycoproteins mZP1–3

Nearly 20 years ago it was reported that the mouse egg ZP (approximately 6.2 μm thick containing approximately 3.5 ng protein) is composed of only three glycoproteins, termed mZP1 (approximately 200 kDa; dimer of identical polypeptides), mZP2 (approximately 120 kDa), and mZP3 (approximately 83 kDa) (Bleil and Wassarman, 1980a; Wassarman *et al.*, 1985; Wassarman, 1988a, 1999). Each of these glycoproteins consists of a unique polypeptide (mZP1, 2 $\times$ approximately 75 kDa; mZP2, approximately 75 kDa; mZP3, approximately 44 kDa) that is heterogeneously glycosylated with both complex-type asparagine-(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 after gel electrophoresis. Each of the polypeptides possesses domains that are conserved among ZP glycoproteins (for example, signal sequences, consensus furin cleavage sites and ZP domains) (Fig. 4).

It is now apparent 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 that have 40–90% similarity) 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

Fig. 2. Schematic diagram of the order of events during fertilization of mammalian eggs. (i) Binding of acrosome-intact spermatozoon to the zona pellucida, (ii) initiation of the acrosome reaction, (iii) penetration of acrosome-reacted spermatozoon through the zona pellucida, (iv) fusion of spermatozoon and egg, (v) the cortical reaction, and (vi) the zona reaction that prevents binding of additional spermatozoa to the zona pellucida. Adapted from Wassarman (1988b).

surrounding eggs from fish, birds, and amphibians contains glycoproteins whose polypeptides resemble mZP1–mZP3. Therefore, there is an evolutionary link between glycoproteins of the vitelline envelope of non-mammalian eggs and glycoproteins of the ZP of mammalian eggs. Since these glycoproteins play essential structural roles in assembly of the extracellular coats during oogenesis (although their synthesis takes place in different sites, for example, in growing oocytes, follicle cells or liver), it is not surprising that they retain common features. It would be of great interest to determine and compare the high resolution structures of vitelline envelope and ZP glycoproteins.

mZP3: a structural glycoprotein

Glycoproteins of the mouse ZP are organized in a very specific manner. The ZP is composed of long interconnected filaments that are polymers of mZP2 and mZP3 (Greve and Wassarman, 1985; Wassarman and Mortillo, 1991). An mZP2–mZP3 dimer is located about every 140 Å along the filaments, thus imposing a structural periodicity that can be

Fig. 3. Light micrograph (dark field) of mouse spermatozoa, eggs and embryos at the end of a competition assay. Spermatozoa are bound to seven ovulated eggs, but are not bound to two two-cell embryos (indicated by arrowheads).

Fig. 4. Schematic diagram of the mZP3 polypeptide showing specific structural and functional domains. 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 zona pellucida.

seen in electron micrographs of dissolved ZP. The filaments, in turn, are crosslinked by mZP1 to create a three-dimensional matrix. Thus, in addition to other functions, each of the ZP glycoproteins plays a structural role during assembly of the ZP. Recently, it was suggested that assembly of the ZP from nascent glycoproteins is a stochastic process that may occur completely outside the growing oocyte (Qi and Wassarman, *in press*).

The mZP3 gene was disrupted by targeted mutagenesis using homologous recombination in mouse embryonic stem cells and null female mice (mZP3^{-/-}) were produced to address certain questions about ZP structure and assembly (Liu *et al.*, 1996; Wassarman *et al.*, 1996). When growing oocytes were isolated from ovaries of juvenile mZP3^{-/-} mice and unfertilized eggs were recovered from adult mZP3^{-/-} mice, they were found to lack a ZP. Furthermore, female mZP3^{-/-} mice were infertile. This observation indicates that in the absence of mZP3 synthesis, a ZP fails to form around growing oocytes during oogenesis, and it emphasizes the critical role played by mZP3 as a structural glycoprotein.

mZP3: a sperm receptor

Only acrosome-intact spermatozoa bind to the ovulated mouse egg ZP. Experimental evidence supports the proposal that, during binding of spermatozoa to eggs, mZP3 serves as a receptor for spermatozoa. For example, of the three glycoproteins that constitute the ZP, only purified mZP3 binds exclusively to the heads of acrosome-intact spermatozoa and thereby prevents them from binding to ovulated eggs *in vitro* (Bleil and Wassarman, 1980b, 1986; Wassarman, 1990; Mortillo and Wassarman, 1991; Wassarman

and Litscher, 1995). Even at nanomolar concentrations, purified unfertilized egg mZP3 is a very effective inhibitor of sperm binding in this competition assay. However, at similar concentrations, mZP3 from fertilized eggs or early embryos has no effect on binding of spermatozoa to eggs *in vitro*. This is consistent with the failure of free-swimming spermatozoa to bind to the ZP of fertilized eggs and preimplantation embryos (Fig. 3). It can be concluded from these and other observations (for example, see Bleil and Wassarman, 1980b; Florman and Wassarman, 1985; Wassarman, 1990) that, as a consequence of the zona reaction, mZP3 is altered such that free-swimming spermatozoa can no longer recognize and bind to the glycoprotein.

The ability of mZP3 to act as a sperm receptor *in vitro* is not significantly affected by high temperatures, detergents, denaturants or reducing agents, or limited proteolysis. Even after extensive proteolysis of mZP3, the small glycopeptides produced retain activity as a sperm receptor, although higher than normal concentrations are required (Florman *et al.*, 1983; Florman and Wassarman, 1985). These observations indicate that mZP3 polypeptide does not play a direct role in sperm receptor function. However, there is considerable information to indicate that mZP3 oligosaccharides play a direct role in sperm receptor function. For example, chemical or enzymic 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, 1985) and certain O-linked related oligosaccharides (Litscher *et al.*, 1995) inhibit binding of spermatozoa to eggs *in vitro* at micromolar concentrations. Collectively, these observations indicate that species-specific sperm-egg binding in mammals is a carbohydrate-mediated event. However, the identity of sugars on mZP3 that are recognized by spermatozoa remains an unresolved and controversial issue.

Limited proteolysis (Rosiere and Wassarman, 1992; Litscher and Wassarman, 1996), exon swapping (Kinloch *et al.*, 1995) and site-directed mutagenesis (Kinloch *et al.*, 1995; Chen *et al.*, 1998) have been used to locate essential O-linked oligosaccharides on mZP3 polypeptide. The results of these studies indicate that the essential oligosaccharides are present on just two of five serine residues, Ser-332 and -334, in a region of polypeptide near the C-terminus that is encoded by exon 7 of the mZP3 gene (eight exons) (Fig. 4). Of the five serine residues, only these two are conserved from mouse to human ZP3. Therefore, the numerous amino acid changes neighbouring 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 the species specificity of sperm-egg interactions (Wassarman and Litscher, 1995).

mZP3: an acrosome reaction inducer

The acrosome is a large secretory vesicle that overlies the nucleus in the apical region of the sperm head (Yanagimachi, 1994). 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 observed as multiple fusions between outer acrosomal membrane and plasma membrane at the anterior region of the sperm head, extensive formation of hybrid membrane vesicles, and exposure of the inner acrosomal membrane and acrosomal contents. Only acrosome-reacted spermatozoa can penetrate the ZP and fuse with the plasma membrane of the egg.

There are many different inducers of the AR (Yanagimachi, 1994). However, it is generally accepted that ZP3 is the natural agonist that initiates the AR upon binding of acrosome-intact spermatozoa to the ZP (Bleil and Wassarman, 1983; Darszon *et al.*, 1996; Wassarman and Florman, 1997; Florman *et al.*, 1998). Criteria are now available that permit the so-called spontaneous AR and the ZP3-induced AR (for example, sensitivity to pertussis toxin) to be distinguished. Although purified mZP3 and large mZP3 glycopeptides induce spermatozoa to undergo the AR *in vitro*, small mZP3 glycopeptides and purified mZP3 O-linked oligosaccharides bind to spermatozoa and inhibit binding to the egg, but do not induce the AR (Wassarman, 1988a, 1990). Leyton and Saling (1989) reported that crosslinking of small mZP3 glycopeptides bound to a spermatozoon can induce the spermatozoon to undergo the acrosome reaction. These findings indicate that induction of the AR by ZP3 may be dependent on multivalent interactions.

Concluding remarks

There is great diversity in the mechanisms of species-specific fertilization. For example, some non-mammalian eggs lack an extracellular coat (for example nematode eggs), while others have a vitelline envelope and a jelly coat (for example echinoderm and amphibian eggs). In some species, the spermatozoon enters the egg at a particular site (micropyle) without the need for an AR (for example nematodes), whereas in others, spermatozoa undergo the AR upon binding to the jelly coat and then bind in a species-specific manner to the vitelline envelope (for example echinoderms and amphibians). It is possible to consider the ZP, which is unique to all mammalian eggs, as an amalgamation of the jelly coat and the vitelline envelope of some non-mammalian eggs. The ZP is the site of both species-specific sperm receptors and inducers of the AR.

In this paper, the current knowledge of the functions of ZP glycoprotein mZP3 in fertilization has been discussed. Three main conclusions can be made: (i) mZP3 is a structural glycoprotein that is required for assembly of the ZP during oogenesis; (ii) mZP3 is a sperm receptor that supports binding of free-swimming spermatozoa to unfertilized eggs; and (iii) mZP3 is an inducer of the AR for spermatozoa bound to the ZP of unfertilized eggs.

Although a great deal is known about mZP3, many more questions remain. A pressing issue concerns the nature of the protein associated with the plasma membrane surrounding the sperm head that recognizes and binds to mZP3 oligosaccharides (so-called egg-binding protein). To date, this area of research, although extremely productive, is

inconclusive (Wassarman, 1999). A second issue concerns the nature of the ZP3 oligosaccharides that are recognized by egg-binding protein. Very little is known about the structure of these oligosaccharides (for example composition, sequence, and conformation) or their sites on ZP3 polypeptides from different mammalian eggs. Can these oligosaccharides account for the species-specific binding of spermatozoa that is observed experimentally? Finally, the mechanism for inactivation of mZP3 as a sperm receptor after fertilization remains to be elucidated. It is hoped that answers to these questions will be forthcoming and that such progress will be of some benefit to clinicians involved in the manipulation of human reproduction.

For more than two decades I have collaborated with excellent graduate students, postdoctoral fellows, and research assistants, first at Harvard Medical School (1972–1985), then at the Roche Institute of Molecular Biology (1986–1995), and now at Mount Sinai School of Medicine (1996–present). I am very grateful to these scientists for their many important contributions to our research on mammalian fertilization. Our research was supported in large part by the National Institute of Child Health and Human Development, National Science Foundation, and Hoffman-LaRoche Inc. Finally, I thank Brian Cook and his staff for their extraordinary hospitality during my stay in Glasgow.

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