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Review

Mouse zona pellucida proteins as receptors for binding of sperm to eggs

Paul M. Wassarman* and Eveline S. Litscher#

Department of Cell, Developmental, and Regenerative Biology, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, New York, NY 10029, USA.

ABSTRACT

Fertilization in mammals is initiated by species-restricted binding of free-swimming sperm to the unfertilized egg's thick extracellular matrix, the zona pellucida (ZP). Both acrosome-intact and acrosome-reacted sperm can bind to the ZP, but only the latter can penetrate the ZP, reach the egg's plasma membrane, and fuse with plasma membrane (fertilization) to produce a zygote. Following fertilization, the ZP is modified by cortical granule components such that acrosome-intact and acrosome-reacted sperm are unable to bind to fertilized eggs. Here we review some of the evidence that bears directly on the involvement of two mouse ZP proteins, mZP2 and mZP3, as receptors for binding of mouse sperm to unfertilized eggs and address some contentious issues surrounding this important initial step in the process of mammalian fertilization.

KEYWORDS: mouse, sperm, eggs, fertilization, ZP proteins, ZP oligosaccharides, sperm binding, capacitation, acrosome reaction, cortical reaction, zona reaction.

ABBREVIATIONS

aa, amino acid; AI, acrosome-intact; AR, acrosome-reacted; C-terminus, carboxy-terminus; Cys, cysteine; IAM, inner acrosomal membrane; Ig, immunoglobulin; kDa, kilodaltons; N-linked, asparagine-linked; N-terminus, amino-terminus; O-linked, serine/threonine-linked; OAM, outer acrosomal membrane; ZP, zona pellucida.

*paul.wassarman@mssm.edu

#eveline.litscher@mssm.edu

INTRODUCTION

1. Mammalian fertilization

Virtually all multicellular organisms are capable of sexual reproduction, a process involving two kinds of gametes, the female's eggs and the male's sperm [1-7]. Egg and sperm fuse with each other (fertilization) to produce a one-cell embryo or zygote – the true beginning of a new individual of the species. The Italian biologist Lazzaro Spallanzani (1729-1799) proposed in 1786 that fertilization requires both sperm and eggs, but it took nearly another century before the process of fertilization was accurately described by the Swiss biologist Herman Fol (1845-1892) and the German biologist Oscar Hertwig (1849-1922).

The process of fertilization in mammals begins when free-swimming sperm bind to the unfertilized egg's thick extracellular matrix, the zona pellucida (ZP), in the ampulla region of the female's oviduct. The ZP was first identified and named by the Baltic German embryologist Karl Ernst von Baer (1792-1876) when describing human eggs in 1827 [8]. The mouse (*Mus musculus*) ZP consists of three heterogeneously glycosylated proteins, called mZP1-3, that are assembled into a crosslinked fibrillar matrix [9, 10]. At the time of fertilization, ovulated

mouse eggs, ≃80 μm in diameter, have exuded a first polar body with separation of chromosomes and are arrested at metaphase II of meiosis. Sperm bind in a species-restricted manner to the egg's ZP (Figure 1), penetrate through the ZP, and one sperm manages to fuse with the egg's plasma membrane. The fertilized egg exudes a second polar body with separation of chromatids and a diploid zygote is the result. Following fertilization, the ZP is subjected to physical and biological changes such that sperm are no longer able to bind to the zygote's ZP. A ZP remains around the cleavage-stage embryo until the expanded blastocyst stage, days 5-6 post-fertilization in mice, when the embryo hatches from the ZP and implants in the uterus.

Several proteins directly involved in mammalian fertilization, such as Izumo (≃39 kDa), an Ig-like protein on acrosome-reacted (AR) sperm, and Juno (≃28 kDa), a glycosylphosphatidylinositol-(GPI-) anchored protein on ovulated eggs, have been identified and characterized in recent years [11-14]. Juno and Izumo serve as binding partners when AR sperm recognize and fuse with unfertilized eggs. However, the nature of the fusogen responsible for sperm-egg fusion has remained elusive [14, 15]. On the other hand, two mouse ZP proteins, mZP2 (≃120 kDa) and mZP3 (≃83 kDa), have been identified as putative receptors for binding of free-swimming mouse sperm to the ZP of unfertilized mouse eggs [16-18]. There is experimental evidence to suggest that both acrosome-intact (AI) and AR mouse sperm can bind to the ZP of unfertilized mouse eggs. Furthermore, it has been proposed that AI sperm bind to mZP3 and AR sperm bind to mZP2. Following fertilization, as part of the block to polyspermy, neither mZP2 nor mZP3 supports binding of supernumerary sperm to the ZP.

Here we review some of the evidence that bears on the involvement of ZP proteins as receptors for binding of mouse sperm to the unfertilized egg's ZP and address some contentious issues surrounding this vital initial step in the fertilization pathway. It is concluded that for mice there are two routes of entrance into the fertilization pathway dependent on two different egg ZP proteins, mZP2 and mZP3, and on the status of the sperm's acrosome, AI or AR.

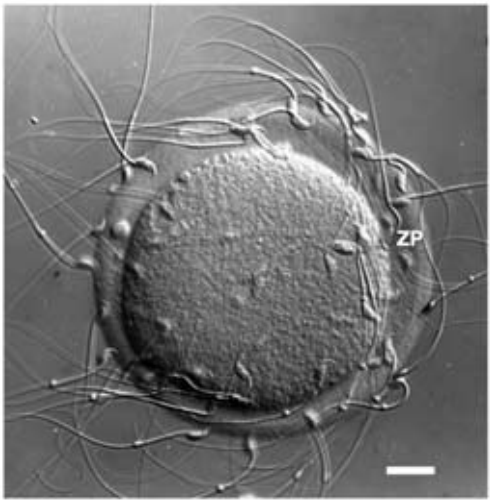

Figure 1. Photographic image of a light micrograph (Nomarski differential interference contrast) of an unfertilized mouse egg incubated in the presence of free-swimming mouse sperm. Sperm are bound to the zona pellucida (ZP). Scale bar (≃1 cm) = ≃14 μm.

2. Sperm capacitation and acrosome reaction

Following release from seminiferous tubules and migration to the epididymis, sperm become highly motile cells [19, 20]. Motility is dependent on many genes that affect flagella structure, as well as on pH, Ca^{2+}, the phospholipase C (PLC) pathway, and the protein kinase A (PKA) pathway activated by cyclic adenosine monophosphate (cAMP). It remains for sperm to undergo the process of capacitation, whereby sperm ejaculated into the female reproductive tract acquire the ability to (1) bind to and penetrate the egg's ZP, (2) complete the acrosome reaction, and (3) fuse with the egg's plasma membrane. During capacitation, efflux of cholesterol from the sperm's plasma membrane takes place and results in a change in membrane fluidity, rearrangement and/or modification of membrane lipids and proteins, and activation of signal transduction by protein kinases. As a consequence of capacitation sperm motility becomes hyperactivated.

The acrosome is a membrane-enclosed, lysosome-like vesicle derived from Golgi during spermiogenesis that sits over the sperm's nucleus in the apical region of the head [20]. The acrosome contains hydrolytic enzymes, such as acrosin, that together with the sperm's motile

force enable bound sperm to penetrate the ZP. Outer acrosomal membrane (OAM) underlies the sperm's plasma membrane and inner acrosomal membrane (IAM) overlies the nucleus. Sperm undergo the acrosome reaction, a form of cellular exocytosis, that involves multiple fusions between OAM and plasma membrane at the anterior region of the sperm's head, extensive formation of hybrid membrane vesicles, and exposure of IAM and acrosomal contents (Figure 2). The acrosome reaction involves a pH increase due to a sperm-specific Na^+/H^+ exchanger, a negative membrane potential due to a sperm-specific K^+ channel, synthesis of cAMP by a soluble adenyl cyclase, alkaline activation of a pH sensitive Ca^{2+} channel, called CatSper (cation channel sperm-associated protein), and a rapid Ca^{2+} influx [21] CatSper is only expressed in males and either inactivation or mutation of the CatSper gene results in sperm with motility defects such that they are unable to fertilize eggs [22]. Only AR sperm can penetrate the egg's ZP, reach the space between the ZP and plasma membrane (perivitelline space), and use plasma membrane remaining over the equitorial segment of the sperm to fuse with the egg's plasma membrane and produce a zygote.

3. Binding of sperm to the egg's ZP

Several studies employing isolated gametes suggest that only AI sperm are able to bind to the egg's ZP and binding causes sperm to undergo the acrosome reaction [23-25]. However, substantial evidence currently supports the view that both AI and AR sperm can bind to the ZP of eggs from various mammals, including mice and humans [26, 27]. It is likely that AI sperm undergo the acrosome reaction either (1) after binding to the ZP [25, 28-32] or (2) while penetrating cumulus cells (*cumulus oophorous*) that surround many unfertilized eggs or while migrating in the oviduct [33-38] and may be species dependent. In this scheme, AI sperm bind to the ZP using plasma membrane that overlies the sperm's head and AR sperm bind to the ZP using IAM exposed during the acrosome reaction. It is reasonable to assume that the two different sperm membranes, plasma membrane overlying the heads of AI sperm and IAM exposed on AR sperm following the acrosome reaction, recognize and bind to different protein epitopes on the egg's ZP. Once fertilization has occurred, neither AI nor AR sperm bind to the ZP of eggs or cleavage-stage embryos (Figure 3).

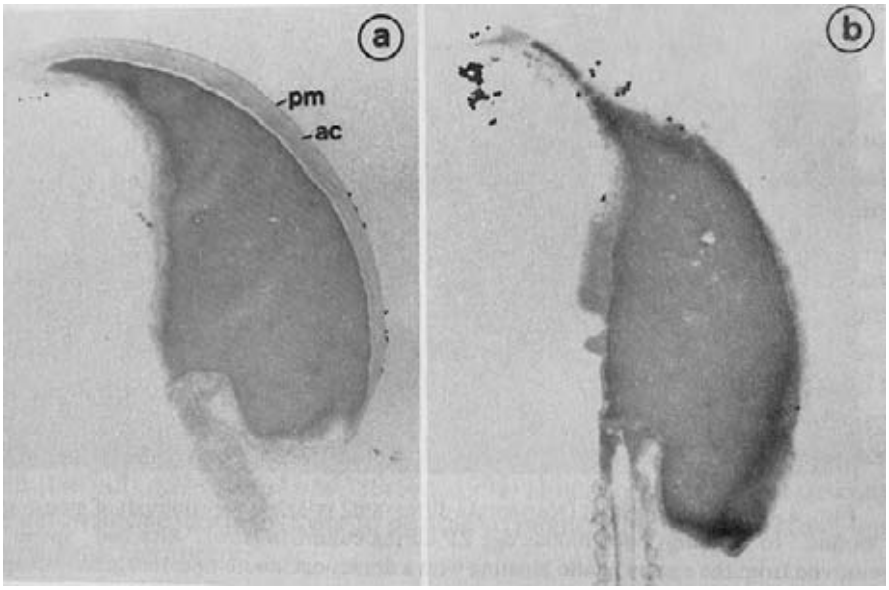

Figure 2. Transmission electron micrographs of thin sections of mouse sperm. Panel A: An acrosome intact (AI) sperm head (pm, plasma membrane; ac, acrosome). Panel B: An acrosome reacted (AR) sperm head. The black dots associated with sperm heads are gold particles added to the preparations.

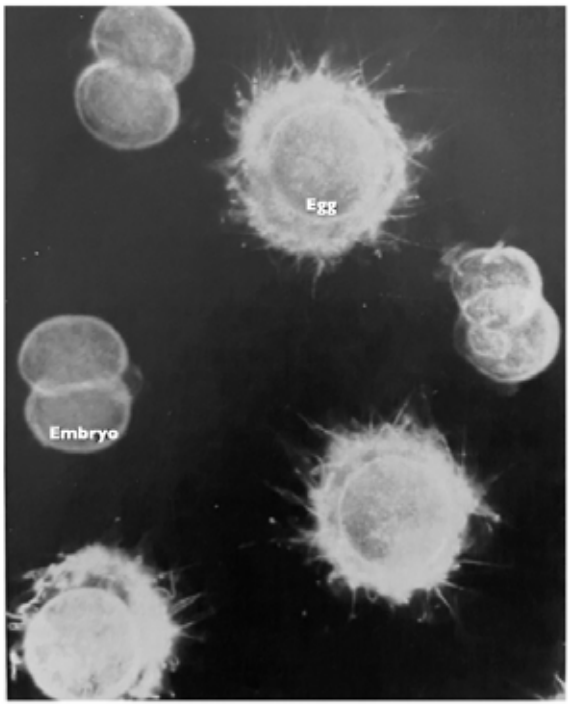

Figure 3. Light micrograph (dark field) of mouse sperm, eggs, and 2-cell embryos cultured *in vitro*. Sperm are bound to the ZP of unfertilized eggs, but not to the ZP of 2-cell embryos due to the zona reaction following fertilization. Sperm do not bind to the ZP of fertilized or artificially activated eggs.

4. Mouse ZP proteins mZP1-3

The ZP of mouse eggs is ≃6 μm thick and consists of a unique set of three heterogeneously glycosylated proteins, called mZP1-3, that differ from proteins present in somatic cell extracellular matrix [9, 10, 39-41]. ZP proteins from different mammals are well conserved, exhibiting ≃60-98% sequence identity. mZP2 (≃120 kDa, 601 aa) and mZP3 (≃83 kDa, 331 aa) are monomers, and mZP1 (≃200 kDa, 528 aa) is a dimer of identical polypeptides connected by an intermolecular disulfide (Figure 4A). The human egg ZP is ≃20 μm thick and is composed of hZP1 (≃200 kDa, 530 aa), hZP2 (≃120 kDa, 604 aa), and hZP3 (≃58 kDa, 330 aa), but has a fourth ZP protein, hZP4 (≃65 kDa, 448 aa), that is a monomer or a dimer stabilized by non-covalent interactions [42]. ZP proteins possess both asparagine (N)-linked and serine/threonine (O)-linked oligosaccharides some of which are sialylated and sulfated [43, 44].

mZP1-3 are secreted proteins synthesized during oogenesis by growing oocytes as precursor polypeptides that have a signal-sequence at the N-terminus and a propeptide at the C-terminus that are cleaved from nascent protein (Figure 4B). Each protein has a ZP domain (ZPD) made up of ≃270 aa, 8 or 10 conserved cysteine (Cys) residues present as intramolecular disulfides, and consists of two subdomains, ZP-N (≃100 aa) and ZP-C (≃135-150 aa) [45-47]. mZP1, hZP1, and hZP4 polypeptides possess a trefoil domain and mZP1, mZP2, hZP1, hZP2, and hZP4 have one or more extra ZP-N subdomains at the N-terminus of their polypeptides [48]. Both subdomains adopt Ig-like folds and in the ZPD are connected to each other by a short, protease senstive linker-region (≃25-30 aa) [49]. ZP-N participates in polymerization of nascent ZP proteins into long fibrils, ≃70-80 Å in width, that are composed of mZP2-mZP3 heterodimers and exhibit a ≃140-150 Å structural repeat [50-53]. Mouse ZP fibrils are crosslinked by mZP1 or, in the case of human ZP fibrils, by hZP1 and possibly hZP4 [54].

Homozygous null female mice completely lacking either mZP2 or mZP3 fail to produce a ZP around growing oocytes, their eggs lack a ZP, and the mice are infertile [55-57]. Infertility is due to a paucity of growing oocytes and Graafian follicles in ovaries that results in a scarcity of ovulated eggs in oviducts. Homozygous knockout female mice lacking mZP1 have an abnormal ZP around oocytes and eggs and exhibit reduced fertility [58]. The latter is due to insufficient crosslinking of ZP fibrils that results in loss of cleavage-stage embryos as they traverse the female reproductive tract on their way to the uterus to implant. Various point, missense, or frameshift mutations in human ZP genes can lead to deleterious effects on ZP formation and to female infertility [10, 59].

5. Binding of mouse AI sperm to mZP3

The notion that the egg's ZP harbors species-restricted receptors for sperm dates back more than a century. In the 1970s it was reported that exposure of mouse sperm to solubilized egg ZP preparations prevented binding of the sperm to ovulated eggs *in vitro* [1, 60]. Similar results were obtained with solubilized hamster egg ZP and sperm. It was concluded that solubilized

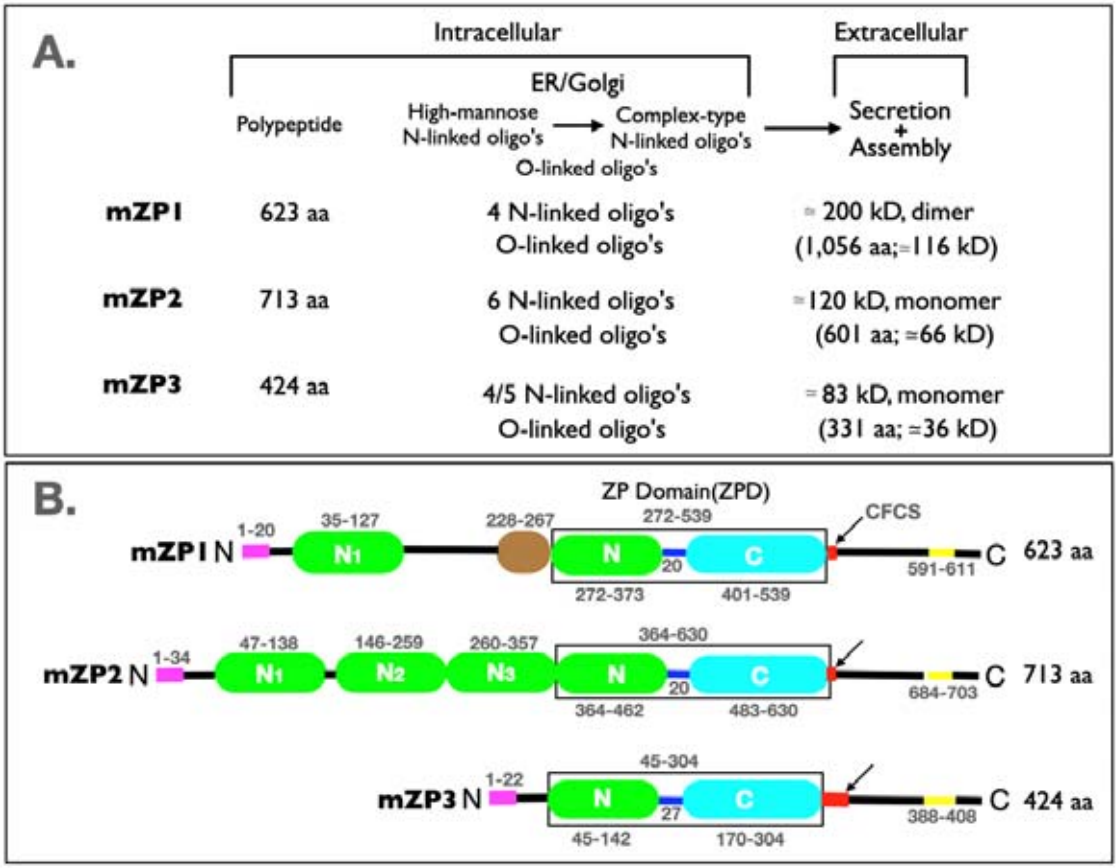

Figure 4. Panel A: Intracellular and extracellular processing of mouse ZP proteins mZP1-3. mZP1 is a dimer and mZP2 and mZP3 are monomers to which high-mannose type N-linked oligosaccharides are added and then converted to complex-type N-linked oligosaccharides intracellularly prior to secretion. In addition, O-linked oligosaccharides are added intracellularly prior to secretion. Nascent ZP polypeptides are processed (e.g., removal of the N-terminal signal-sequence as nascent proteins move from the endoplasmic reticulum to the Golgi), packaged into large secretory vesicles originating from the Golgi that move to the plasma membrane, cleaved at the consensus furin cleavage-site (removal of the C-terminal propeptide), secreted, and assembled into long fibrils extracellulary. The average molecular weights for secreted mZP1-3 are shown, based on electrophoretic mobility on SDS-gels, together with the sizes (number aa) of mZP1-3 polypeptides. Note that the apparent molecular weights of mZP1-3 are significantly higher than the sizes of the polypeptides due to the addition of N- and O-linked oligosaccharides. Panel B: Schematic representation of the organization of mouse ZP proteins, mZP1–3 (623, 713, and 424 amino acids, respectively). The polypeptides contain a signal sequence (SS) at the N-terminus (pink), a ZP domain (ZPD; black box) consisting of ZP-N (green) and ZP-C (turquoise) subdomains and a short linker region (blue), and a consensus furin cleavage-site (CFCS; arrow), transmembrane domain (TMD; yellow), and C-terminal propeptide (CTP). mZP1 has a trefoil domain (brown) adjacent to the ZPD. mZP1 and mZP2 have one or three extra copies of the ZP-N subdomain (green) between the N-terminus of the polypeptides and the ZPD. The amino acid numbers for each region of the mouse ZP polypeptides are indicated above and below the drawings of the polypeptides.

mammalian ZP contained sperm receptors that bound to sperm and prevented them from binding to eggs.

In the early 1980s it was reported that exposure of AI mouse sperm to solubilized egg ZP prevented binding of the sperm to ovulated eggs *in vitro* (≃80% inhibition at 4 ZP/µl) [16]. Solubilized 2-cell embryo ZP had no effect on sperm binding to eggs. Moreover, purified egg mZP3, at nanomolar concentrations, prevented binding of sperm to ovulated eggs (≃60% inhibition at 4 ZP equivalents/µl), whereas purified egg mZP1 and

mZP2 had no significant effect on sperm binding to eggs. Furthermore, AI sperm exposed to purified egg mZP3 were induced to undergo the acrosome reaction *in vitro* [28]. mZP3 purified from 2-cell embryo ZP had no effect on binding of AI sperm to eggs and did not induce the acrosome reaction, consistent with observations that sperm do not bind to the ZP of fertilized eggs or cleavage-stage embryos. These findings suggested that egg mZP3 is a receptor for AI sperm.

Subsequently, a solid-phase assay, employing silica beads to which mZP3 was covalently linked (mZP3-beads), was used to evaluate sperm binding [61]. It was found that AI sperm bind by their head specifically to mZP3-beads (usually one sperm bound per bead), but not to beads derivatized with mZP2, fetuin, or serum albumin (Figure 5). Over time at room temperature or an earlier time at 37 ^{o}C, AI sperm bound to mZP3-beads underwent the acrosome reaction (AR sperm) and were released from the beads. Inhibitors of the ZP induced acrosome reaction (e.g., islet-activating protein and 3-quinuclidinyl benzilate) prevented sperm bound to mZP3-beads from undergoing the acrosome reaction. In other studies, binding of egg mZP3 to AI sperm heads was visualized by either whole-mount autoradiography or transmission electron microscopy using either radiolabeled- (^{125}I-) mZP3 or colloidal gold-labeled mZP3, respectively [62, 63]. Radiolabeled or gold-labeled fetuin, a protein that possesses N- and O-linked oligosaccharides, was used as a control in the experiments. In both protocols egg mZP3 was found localized at high levels to plasma membrane associated with the acrosomal cap region of AI sperm heads, but at nearly background levels with IAM of AR sperm heads or with sperm tails (Figures 6 and 7). Fetuin was present on AI and AR sperm at very low levels. These and other observations are consistent with the suggestion that mZP3 present in the mouse egg's ZP is a receptor for AI sperm.

6. Binding of mouse AI sperm to mZP3 oligosaccharides

A variety of lectins (e.g., *Ricinus communis* agglutinin, RCA1; concanavalin A, ConA; wheat germ agglutinin, WGA) that bind to oligosaccharides and antibodies raised against

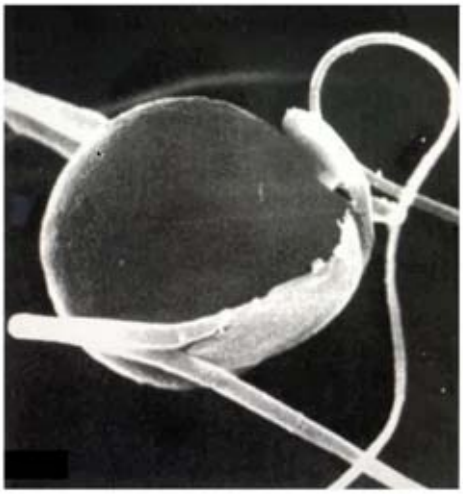

Figure 5. Scanning electron micrograph of acrosome-intact (AI) sperm bound to an mZP3-bead. Styrene beads were derivatized with mZP3 and then incubated with capacitated AI sperm. Shown is an example of sperm bound to an mZP3-bead. For further information see [61].

oligosaccharides bind to the ZP of mammalian eggs [64]. Certain lectins (e.g., WGA) can prevent fertilization and some lectin binding sites (e.g., RCA1) are more densely distributed in the exterior region of the ZP [65]. In this context, the molecular weight ($\simeq$83 kDa) and isoelectric point (pI 4.2-5.2) of purified mZP3 exhibit considerable heterogeneity due largely to the presence of N- and O-linked oligosaccharides, some of which are sialylated or sulfated (i.e., negatively charged) [41]. Nascent mZP3 has as many as five high-mannose type N-linked oligosaccharides (concensus sequence Asn-X-Ser/Thr) that are converted to complex-type prior to secretion and assembly into ZP fibrils [66-68]. Complex-type N-linked oligosaccharides often have N-acetylglucosamine, galactose, fucose, and sialic acid as terminal residues. mZP3 also has an undetermined number of core type-1 and type-2 O-linked oligosaccharides (no concensus sequence) that are terminated with N-acetylglucosamine, galactose, and sialic acid.

Several observations suggest that AI mouse sperm bind to mZP3 oligosaccharides rather than to polypeptide [69-72]. For example, exposure of purified mZP3 to high temperatures, proteases, denaturants, detergents, or fixatives has little to no effect on its ability to bind to AI sperm.

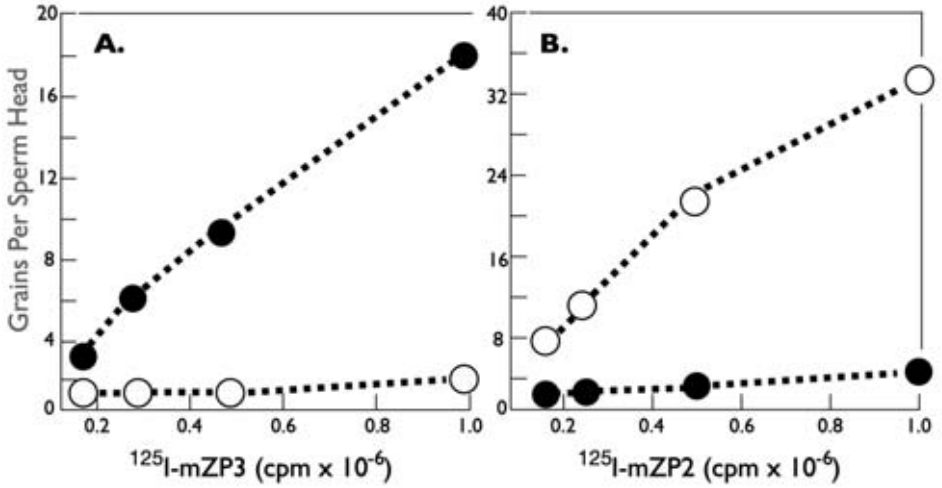

Figure 6. Quantitation of mZP2, mZP3, and fetuin radiolabeled-probes bound to acrosome-intact (AI) and acrosome-reacted (AR) sperm. Panel A: Binding of radiolabeled mZP3 (^{125}I-mZP3) to acrosome-intact (AI; closed circles) and acrosome-reacted (AR; open circles) mouse sperm as a function of probe concentration. Panel B: Binding of radiolabeled mZP2 (^{125}I-mZP2) to AI (closed circles) and AR (open circles) mouse sperm as a function of probe concentration. Radiolabeled fetuin (^{125}I-fetuin) binding to AI and AR sperm occurred at insignificant levels. It is clear that mZP3 binds preferentially to AI sperm and mZP2 binds preferentially to AR sperm. For further information see [62].

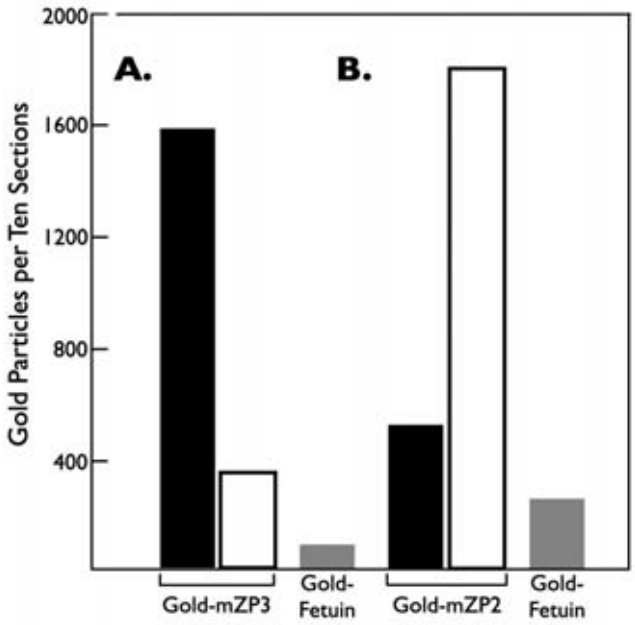

Figure 7. Quantitation of mZP2, mZP3, and fetuin gold-probes bound to acrosome-intact (AI) and acrosome-reacted (AR) sperm. Panel A: Shown are the average number of 5 mm gold particles present per ten sections of AI sperm (solid bar), AR sperm (open bar), and fetuin (shaded bar) for the gold-mZP3 probe. Panel B: Shown are the average number of 5 mm gold particles present per ten sections of AI sperm (solid bar), AR sperm (open bar), and fetuin (shaded bar) for the gold-mZP2 probe. In each case, sections containing the largest portion of the sperm head were chosen for analysis. Using fetuin gold-probes for assessment of background binding levels, it is clear that mZP3 binds preferentially to AI sperm and mZP2 binds preferentially to AR sperm. For further information see [63].

This indicates that mZP3 is a very stable receptor for AI sperm and suggests that its ability to serve as a receptor is certainly not dependent on polypeptide conformation. In this context, a heavily glycosylated peptide ($\simeq$55 ± 8 kDa) derived from the C-terminal portion of mZP3 by mild proteolytic digestion and small glycopeptides ($\simeq$1.5-6 kDa) derived by extensive proteolytic digestion of mZP3 are as effective as intact mZP3 in preventing binding of AI sperm to eggs *in vitro* [73-75]. Furthermore, exposure of AI sperm to (1) Lewis X (Le*)-containing glycans, (2) branched oligosaccharides (biantennary and tetraantennary) having an α- or β-linked galactose at the non-reducing end (related to blood group B-type oligosaccharides), or (3) O-linked oligosaccharides released from mZP3 by alkaline hydrolysis, prevent binding of sperm to eggs *in vitro* [69, 76-80]. These and other observations suggest that mZP3 oligosaccharides rather than polypeptide are primarily responsible for binding of mZP3 to AI sperm and preventing the sperm from binding to eggs. A similar conclusion was reached upon examination of binding of a mutated form of avian ZP3 deficient in a single O-linked oligosaccharide [81].

Involvement of mZP3 oligosaccharides in binding of sperm to eggs is analogous to their role in binding of sperm from marine animals and amphibians to eggs, sexual agglutination in budding yeast, and binding of bacteria, animal viruses, and other pathogens to their cellular hosts. There also is evidence to suggest that mammalian sperm interact with oligosaccharides on the surface of the oviductal epithelium as sperm migrate toward the oviduct [82, 83]. On the other hand, it was found that female mice deficient in specific glycosyl transferases (homozygous nulls) involved in N- and O-linked oligosaccharide biosynthesis are fertile [84-86]. The findings were interpreted to mean that mZP3 oligosaccharides are not involved in binding of mZP3 to sperm. In such experiments, it would have been of interest to purify mZP3 possessing N- and/or O-linked oligosaccharides deficient in specific sugar moieties (e.g., galactose or N-acetylglucosamine) and assess whether the modified mZP3 binds to AI sperm and prevents their binding to eggs *in vitro*. It is possible that fertilization was achieved in these experiments by binding of AR sperm, not AI sperm, to eggs without the involvement of mZP3.

7. Binding of mouse AR sperm to mZP2 polypeptide

Results of experiments in the 1980s suggested that, while mZP3 serves as a receptor for AI sperm, mZP2 serves as a receptor for AR sperm [87]. Binding of egg mZP2 to AR sperm heads was visualized by either whole-mount autoradiography or transmission electron microscopy using either radiolabeled-(^{125}I-) mZP2 [62] or colloidal gold-labeled mZP2 [63], respectively. As described above, radiolabeled or gold-labeled fetuin was used as a control in the experiments. In both protocols egg mZP2 was found primarily associated at high levels with IAM of AR sperm heads, but at nearly background levels with plasma membrane of AI sperm heads or with sperm tails (Figures 7 and 8). Fetuin was present on AI and AR sperm at very low levels. These observations are consistent with the suggestion that mZP2 present in the mouse egg's ZP is a receptor for AR sperm.

Extensive studies have now been carried out to elucidate the molecular basis of AR sperm binding to mZP2. Overall, results of these studies strongly suggest that (1) AR sperm bind to the N-terminal region of mZP2 (aa 35-149), (2) C-terminal portions of mZP2 are not required for AR sperm binding, and (3) AR sperm binding to mZP2 is not dependent on the protein's N- or O-linked oligosaccharides [18, 88, 89]. Furthermore, proteolytic cleavage of mZP2 within its N-terminal region following sperm-egg fusion precludes binding of AR sperm to mZP2. These observations suggest that AR sperm bind directly to the N-terminus of ZP2 polypeptide prior to fertilization, but are unable to bind to ZP2 following fertilization.

8. ZP block to polyspermy

If more than one sperm fuses with an egg (polyspermy) embryonic death usually occurs. Consequently, following fertilization conditions are established that prevent supernumerary sperm from fusing with fertilized eggs. Mechanisms to prevent polyspermy are activated at the egg's

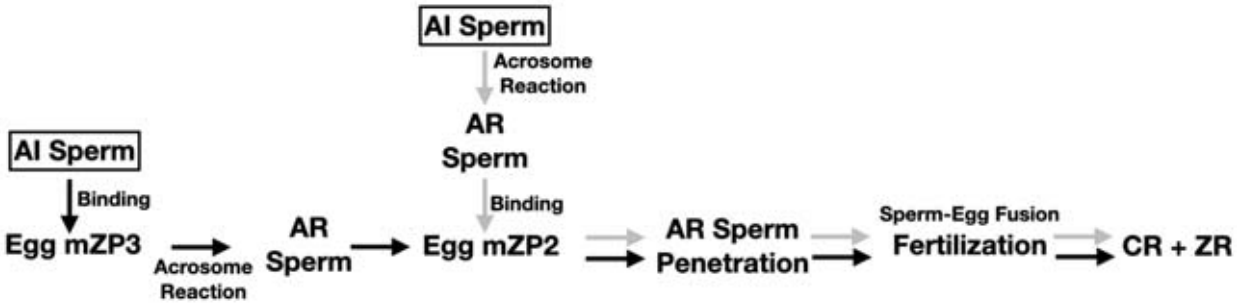

Figure 8. Schematic diagram of some steps involved in two different pathways to fertilization in mice. In the first pathway (black arrows), acrosome-intact (AI) sperm bind to mZP3 in the egg's zona pellucida (ZP). As a result of binding to mZP3, AI sperm undergo the acrosome-reaction (multiple fusions between the sperm's outer acrosomal membrane and plasma membrane leading to exposure of the inner acrosomal membrane), and acrosome-reacted (AR) sperm then bind to mZP2. In the second pathway (gray arrows), AI sperm undergo the AR prior to reaching the ZP, perhaps in the *cumulus oophorous* of ovulated eggs, and then bind to mZP2 rather than to mZP3. In both pathways AR sperm penetrate the ZP and fuse with the egg's plasma membrane (fertilization). Fusion of sperm and egg induces the cortical reaction (CR) in eggs (fusion of cortical granule membrane and the egg's plasma membrane) that results in exocytosis of cortical granule components, including Zn^{2+}, into the ZP and induces the zona reaction (ZR). The ZR consists of inactivation of mZP2 and mZP3 as sperm receptors (AI and AR sperm cannot bind to the ZP of fertilized eggs) and hardening of the ZP.

plasma membrane and the ZP [90, 91]. The former is referred to as the fast block and the latter as the slow block to polyspermy. The precise nature of the fast block in mouse eggs is not completely clear, but may be due to depolarization of plasma membrane (i.e., an electrical change). On the other hand, the nature of the slow block involves changes in the ZP due to the zona reaction, that includes a significant decrease in the solubility of the ZP, the so-called hardening reaction (e.g., $\simeq$2- to 4-fold increase in stiffness and viscosity) [92]. As a result, sperm that had partially penetrated the ZP prior to fertilization can penetrate no further and free-swimming AR and AI sperm can no longer bind to the ZP.

Alteration of the ZP following fertilization is largely attributable to fusion of cortical granules in the egg's cytoplasmic cortex (each mouse egg has $\simeq$ 4,500 cortical granules) with the egg's plasma membrane, the cortical reaction [93, 94]. The latter results in release of cortical granule components into the perivitelline space and then into the relatively porous ZP. The cortical reaction requires translocation of cortical granules to the inner surface of the plasma membrane using myosin IIA and elevation of Ca^{2+} concentration (peaks at $\simeq$1 μM in mouse eggs) due to release of Ca^{2+} stores from endoplasmic reticulum by inositol triphosphate (IP3). Several Ca^{2+}-dependent proteins, including calmodulin, protein kinase C (PKC), Ca^{2+}/calmodulin-dependent protein kinases (CAMKs), synaptotagmins (SYTs), Rab-3, and rabphilin-3A may participate in the cortical reaction. The cortical reaction also results in accumulation of Zn^{2+} in the ZP, referred to as zinc sparks [89, 95, 96], and release of a metalloendoprotease, called ovastacin, that cleaves mZP2 near its N-terminus (166Leu-Ala↓Asp-Glu169) without release of a peptide [88, 97-101]. Proteolytic cleavage of mZP2 is involved in hardening of the ZP and inactivation of mZP2 as a binding partner for AR sperm. High concentrations of Zn^{2+} also have a negative effect on sperm motility, thereby assisting in the prevention of sperm penetration through the ZP and may alter the conformation of ZP crosslinks by binding to specific sites on ZP1. Inactivation of mZP3 as a binding partner for AI sperm also occurs following the cortical reaction, but the molecular basis of inactivation is unclear.

9. Fertilization pathway in mice

In mice it would appear that, depending on the status of the sperm's acrosome, AI or AR sperm, there are two different ways to enter the

fertilization pathway (Figure 8). AI sperm bind to mZP3 in the egg's ZP, undergo the acrosome reaction, and bind to mZP2. On the other hand, sperm that undergo the acrosome reaction prior to reaching the ZP, perhaps in the *cumulus oophorous* surrounding ovulated eggs or in the oviduct, bind directly to mZP2 by their IAM rather than to mZP3. A component(s) of plasma membrane overlying heads of AI sperm and a component(s) of IAM of AR sperm recognize different epitopes on the egg's ZP. In both cases AR sperm penetrate the ZP and fuse with the egg's plasma membrane (fertilization). Fusion of sperm and egg induces the cortical reaction that involves exocytosis of cortical granule components, including ovastacin and Zn^{2+}, into the ZP and induces the zona reaction. The zona reaction causes a decrease in solubility (hardening) of the ZP, as well as inactivation of mZP2 and mZP3 as receptors for AR sperm and AI sperm, respectively. These changes in the ZP help to ensure that polyspermic fertilization does not take place.

10. Final comments

As a consequence of its vital importance in human biology, the process of fertilization has been of intense interest to research scientists for more than a century. During the past 50 years or so, by taking advantage of major advances in genetic, biochemical, and molecular methodology, significant progress has been made in identifying and characterizing mammalian egg and sperm components that participate in mammalian fertilization. As expected, there are differences of opinion about how to interpret certain disparate *in vitro* and *in vivo* results. However, despite the differences, we are now on the road to a much clearer understanding of the molecular basis of many steps in the fertilization pathway.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no disclosures.

REFERENCES

1. Gwatkin, R. B. L. 1977, Fertilization Mechanisms in Man and Mammals. Plenum Press, New York, NY.
2. Yanagimachi, R. 1994, The Physiol. Reprod., 1, 189-317, Raven Press, New York, NY.
3. Wassarman, P. M. 1999, Cell, 96, 175-183.
4. Florman, H. M. and Ducibella, T. 2006, Knobil and Neill's Physiol. Reprod., 1, 149-196, Academic Press/Elsevier, San Diego, CA.
5. Wassarman, P. M. and Vacquier, V. D. (Eds.). 2008, Fertil. Intl. J. Dev. Biol., 52, 409-796.
6. Florman, H. M. and Fissore, R. A. 2015, Knobil and Neill's Physiol. Reprod., 1, 55-112, Academic Press/Elsevier, San Diego, CA.
7. Bhakta, H. H., Refai, F. H. and Avella, M. A. 2019, Development, 146, dev176966.
8. von Baer, K. E. 1827, De Ovi Mammalium et Hominis Genesi (On the Genesis of the Ovum of Mammals and of Man), Leopold Voss, Leipzig, Germany.
9. Wassarman, P. M. and Litscher, E. S. 2018, Curr. Top. Dev. Biol., 130, 331-356.
10. Wassarman, P. M. and Litscher, E. S. 2021, Genes (Basel), 12, 1266.
11. Inoue, N., Ikawa, M., Isotani, A. and Okabe, M. 2005, Nature, 434, 234-238.
12. Bianchi, E., Doe, B., Goulding, D. and Wright, G. J. 2014, Nature, 508, 483-487.
13. Grayson, P. 2015, Roy. Soc. Open Sci., 2, 150296.
14. Bianchi, E. and Wright, G. J. 2020, PLoS Biol., 18, e3000953.
15. Moi, D., Nishio, S., Li X., Valansi, C., Langleib, M., Brukman, N. G., Flyak, K., Dessimoz, C., de Sanctis, D., Tunyasuvunakool, K., Jumper, J., Grana, M., Romero, H., Aguilar, P. S., Jovine, L. and Podbilewicz, B. 2022, Nature Commun., 13, 3880.
16. Bleil, J. D. and Wassarman, P. M. 1980, Cell, 20, 873-882.

17. Bleil, J. D., Greve, J. M. and Wassarman, P. M. 1988, Dev. Biol., 128, 376-385.
18. Avella, M. A., Baibakov, B. and Dean, J. 2014, J. Cell Biol., 205, 801-809.
19. Katz, D. F., Cherr, G. N. and Lambert, H. 1986, Gamete Res., 14, 333-346.
20. Toshimori, K. and Eddy, E. M. 2015, Knobil and Neill's Physiol. Reprod., 1, 99-148, Academic Press/Elsevier, San Diego, CA.
21. Florman, H. M., Jungnickel, M. K. and Sutton, K. A. 2008, Intl. J. Dev. Biol., 52, 503-510.
22. Lishko, P. V., Kirichok, Y., Ren, D., Navarro, B. Chung, J. J. and Clapham, D. E. 2012, Ann. Rev. Physiol., 74, 453-475.
23. Saling, P. M. and Storey, B. T. 1979, J. Cell Biol., 83, 544-554.
24. Saling, P. M., Sowinsky, J. and Storey, B. T. 1979, J. Exp. Zool., 209, 229-238.
25. Florman, H. M. and Storey, B. T. 1982, Dev. Biol., 91, 121-130.
26. Morales, P., Cross, N. L., Overstreet, J. W. and Hanson, F. W. 1989, Dev. Biol., 133, 385-392.
27. Myles, D. G., Hyatt, H. and Primakoff, P. 1987, Dev. Biol., 121, 559-567.
28. Bleil, J. D. and Wassarman, P. M. 1983, Dev. Biol., 95, 317-324.
29. Cherr, G. N., Lambert, H., Meizel, S. and Katz, D. F. 1986, Dev. Biol., 114, 119-131.
30. Cross, N. L., Morales, P., Overstreet, J. W. and Hanson, F. W. 1988, Biol. Reprod., 38, 235-244.
31. Buffone, M. G., Rodriguez-Miranda, E., Storey, B. T. and Gerton, G. L. 2009, J. Cell Physiol., 220, 611-620.
32. Balbach, M., Hamzeh, H., Jikeli, J. F., Brenker, C., Schiffer, C., Hansen, J. N., Neugebauer, P., Trötschel, C., Jovine, L., Han, L., Florman, H. M., Kaupp, U. B., Strünker, T. and Wachten, D. 2020, Front. Cell Dev. Biol., 8, 572735.
33. Jin, M., Fujiwara, E., Kakiuchi, Y., Okabe, M., Satouh, Y., Baba, S. A., Chiba, K. and Hirohashi, N. 2011, Proc. Natl. Acad. Sci. USA, 108, 4892-4896.
34. Inoue, N., Satouh, Y., Ikawa, M., Okabe, M. and Yanagimachi, R. 2011, Proc. Natl. Acad. Sci. USA, 108, 20008-20011.
35. Avella, M. A. and Dean, J. 2011, Proc. Natl. Acad. Sci. USA, 108, 19843-19844.
36. La Spina, F. A., Puga Molina, L. C., Romarowski, A., Vitale, A. M., Falzone, T. L., Krapf, D., Hirohashi, N. and Buffone, M. G. 2016, Dev. Biol., 411, 172-182.
37. Hino, T., Muro, Y, Tamura-Nakano, M., Okabe, M., Tateno, H. and Yanagimachi, R. 2016, Biol. Reprod., 95, 50.
38. Hirohashi, N. and Yanagimachi, R. 2018, Biol. Reprod., 99, 127-133.
39. Bleil, J. D. and Wassarman, P. M. 1980, Dev. Biol., 76, 185-202.
40. Shimizu, S., Tsuji, M. and Dean, J. 1983, J. Biol. Chem., 258, 5858-5863.
41. Wassarman, P. M. 1988, Ann. Rev. Biochem., 57, 415-442.
42. Gupta, S. K. 2018, Curr. Top. Dev. Biol., 130, 379-411.
43. Varki, A., Cummings, R. D., Esko, J. D., Stanley, P., Hart, G. W., Aebi, M., Mohnen, D., Kinoshita, T. and Packer, N. H. (Eds.). 2022, Essentials of Glycobiology, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
44. Vliegenthart, J. F. G. 2017, Proc. Jpn. Acad. Ser B, 93, 64-86.
45. Bork, P. and Sander, C. 1992, FEBS Lett., 300, 237-240.
46. Jovine, L., Darie, C. C., Litscher, E. S. and Wassarman, P. M. 2005, Ann. Rev. Biochem., 74, 83-114.
47. Litscher, E. S. and Wassarman, P. M. 2015, A Guide to Zona Pellucida Domain Proteins, John Wiley and Sons, Hoboken, NJ.
48. Callebaut, I., Mornon, J. P. and Monget, P. 2007, Bioinformatics, 23, 1871-1874.
49. Bokhove, M. and Jovine, L. 2018, Curr. Top. Dev. Biol., 130, 413-442.
50. Greve, J. M. and Wassarman, P. M. 1985, J. Mol. Biol., 181, 253-264.
51. Jovine, L., Qi, H., Williams, Z., Litscher, E. and Wassarman, P. M. 2002, Nature Cell Biol., 4, 457-461.
52. Jovine, L., Janssen, W. G., Litscher, E. S. and Wassarman, P. M. 2006, BMC Biochem., 7, 11.
53. Litscher, E. S. and Wassarman, P. M. 2020, Ann. Rev. Biochem., 89, 695-715.

54. Nishimura, K., Dioguardi, E., Nishio, S., Villa, A., Han, L. and Jovine, L. 2019, Nature Commun., 10, 3086.
55. Liu, C., Litscher, E. S., Mortillo, S., Sakai, Y., Kinloch, R. A., Stewart, C. L. and Wassarman, P. M. 1996, Proc. Natl. Acad. Sci, USA, 93, 5431-5436.
56. Rankin, T., Familiari, M. Lee, E., Ginsberg, A., Dwyer, N., Blanchette-Mackie, J., Drago, J., Westphal, H. and Dean, J. 1996, Development, 122, 2903-2910.
57. Rankin, T. L., O'Brien, M., Lee, E., Wigglesworth, K., Eppig, J. and Dean, J. 2001, Development, 128, 1119-1126.
58. Rankin, T., Talbot, P., Lee, E. and Dean, J. 1999, Development, 126, 3847-3855.
59. Wassarman, P. M. and Litscher, E. S. 2022, eLife, 11, e76106.
60. Gwatkin, R. B. L. and Williams, D. T. 1976, J. Reprod. Fertil., 49, 55-59.
61. Vazquez, M. H., Phillips, D. M. and Wassarman, P. M. 1989, J. Cell Sci., 92, 713-722.
62. Bleil, J. D. and Wassarman, P. M. 1986, J. Cell Biol., 102, 1363-1371.
63. Mortillo, S. and Wassarman, P. M. 1991, Development, 113, 141-149.
64. Oikawa, T., Yanagimachi, R. and Nicolson, G. L. 1973, Nature, 241, 256-259.
65. Nicolson, G. L., Yanagimachi, R. and Yanagimachi H. 1975, J. Cell Biol., 66, 263-274.
66. Greve, J. M., Salzmann, G. S., Roller, R. J. and Wassarman, P. M. 1982, Cell, 31, 749-759.
67. Salzmann, G. S., Greve, J. M, Roller, R. J. and Wassarman, P. M. 1983, EMBO J., 2, 1451-1456.
68. Boja, E. S., Hoodbhoy, T., Fales, H. M. and Dean, J. 2003, J. Biol. Chem. 278, 34189-34202.
69. Wassarman, P. M. 1989, Ciba Found. Symp., 145, 135-155.
70. Dell, A., Morris, H. R., Easton, R. L., Patankar, M. and Clark, G. F. 1999, Biochim. Biophys. Acta, 1473, 196-205.
71. Visconti, P. E. and Florman, H. M. 2010, Science Signaling 3, 1-3.
72. Pang, P-C., Chiu, P. C. N., Lee, C-L., Chang, L-Y., Panico, M., Morris, H. R., Haslam, S. M., Khoo, K-H., Clark, G. F., Yeung, W. S. B. and Dell, A. 2011, Science, 333, 1761-1764.
73. Florman, H. M., Bechtol, K. B. and Wassarman, P. M. 1984, Dev. Biol., 106, 243-255.
74. Rosiere, T. K. and Wassarman, P. M. 1992, Dev. Biol., 154, 309-317.
75. Litscher, E. S. and Wassarman, P. M. 1996, Biochemistry, 35, 3980-3985.
76. Florman, H. M. and Wassarman, P. M. 1985, Cell, 41, 313-324.
77. Litscher, E. S., Juntunen, K., Seppo, A., Penttila, L., Niemela, R., Renkonen, O. and Wassarman, P. M. 1995, Biochemistry, 34, 4662-4669.
78. Johnston, D. S., Wright, W. W., Shaper, J. H., Hokke, C. H., Van den Eijnden, D. H. and Joziasse, D. H. 1998, J. Biol. Chem., 273, 1888-1895.
79. Kerr, C. L., Hanna, W. F., Shaper, J. H. and Wright, W. W. 2002, Biol. Reprod., 66, 1585-1595.
80. Kerr, C. L., Hanna, W. F., Shaper, J. H. and Wright, W. W. 2004, Biol. Reprod., 71, 770-777.
81. Han, L., Monné, M., Okumura, H., Schwend, T., Cherry, A. L., Flot, D., Matsuda, T. and Jovine, L. 2010, Cell, 143, 404-415.
82. Machado, S. A., Kadirvel, G., Daigneault, B. W., Korneli, C., Miller, P., Bovin, N. and Miller, D. J. 2014, Biol. Reprod., 91, 1-9.
83. Suarez, S. S. 2016, Cell Tiss. Res., 363, 185-194.
84. Thall, A. D., Maly, P. and Lowe, J. B. 1995, J. Biol. Chem., 270, 21437-21440.
85. Shi, S, Williams, S. A., Seppo, A., Kurniawan, H., Chen, W., Ye, Z., Marth, J. D. and Stanley, P. 2004, Mol. Cell. Biol., 24, 9920-9929.
86. Williams, S. A., Xia, L., Cummings, R. D., McEver, R. P. and Stanley, P. 2007, J. Cell Sci., 120, 1341-1349.
87. Bleil, J. D. and Wassarman, P. M. 1988, Proc. Natl. Acad. Sci. USA, 85, 6778-6782.

88. Gahlay, G., Gauthier, L., Baibakov, B., Epifano, O. and Dean, J. 2010, Science, 329, 216-219.
89. Tokuhiro, K. and Dean, J. 2018, Dev. Cell, 46, 627-640.
90. Evans, J. P. 2020, Mol. Reprod. Dev., 87, 341-349.
91. Fahrenkamp, E., Algarra, B. and Jovine, L. 2020, Mol. Reprod. Dev., 87, 326-340.
92. Kim, J. and Kim, J. 2013, IEEE Trans. Biomed. Eng., 60, 569-575.
93. Ducibella, T. 1996, Human Reprod. Update, 2, 29-42.
94. Liu, M. 2011, Reprod. Biol. Endocrinol., 9, 149.
95. Kim, A. M., Bernhardt, M. L., Kong, B. Y., Ahn, R. W., Vogt, S., Woodruff, T. K. and O'Halloran, T. V. 2011, ACS Chem. Biol., 6, 716-723.
96. Que, E. L., Duncan, F. E., Bayer, A. R., Philips, S. J., Roth, E. W., Bleher, R., Gleber, S. C., Vogt, S., Woodruff, T. K. and O'Halloran, T. V. 2017, Itegr. Biol. (Camb.), 9, 135-144.
97. Bleil, J. D., Beall, C. F. and Wassarman P. M. 1981, Dev. Biol., 86, 189-197.
98. Moller, C. C. and Wassarman, P. M. 1989, Dev. Biol., 132, 103-112.
99. Quesada, V., Sánchez, L. M., Alvarez, J. and López-Otin, C. 2004, J. Biol. Chem., 279, 26627-26634.
100. Burkart, A. D., Xiong, B., Baibakov, B., Jimenez-Movilla, M. and Dean, J. 2012, J. Cell Biol., 197, 37-44.
101. Körschgen, H., Kuske, M., Karmilin, K., Yiallouros, I., Balbach, M. Floehr, J., Wachten, D., Jahnen-Dechent, W. and Stöcker, W. 2017, Mol. Hum. Reprod., 23, 607-616.