Focus on Fertilization

Insights into the molecular basis of sperm–egg recognition in mammals

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Abstract

The zona pellucida surrounding the egg and pre-implantation embryo is required for in vivo fertility and early development. Explanatory models of sperm–egg recognition need to take into account the ability of sperm to bind to ovulated eggs, but not to two-cell embryos. For the last two decades, investigators have sought to identify an individual protein or carbohydrate side chain as the ‘sperm receptor’. However, recent genetic data in mice are more consistent with the three-dimensional structure of the zona pellucida, rather than a single protein (or carbohydrate), determining sperm binding. The mouse and human zonae pellucidae contain three glycoproteins (ZP1, ZP2, ZP3) and, following fertilization, ZP2 is proteolytically cleaved. The replacement of endogenous mouse proteins with human ZP2, ZP3 or both does not alter taxon specificity of sperm binding or prevent fertility. Surprisingly, human ZP2 is not cleaved following fertilization and intact ZP2 correlates with persistent sperm binding to two-cell embryos. Taken together, these data support a model in which the cleavage status of ZP2 modulates the three-dimensional structure of the zona pellucida and determines whether sperm bind (uncleaved) or do not (cleaved).


Introduction

Vertebrate eggs are surrounded by an extracellular matrix variously known as the vitelline coat (amphibians), chorion (teleosts), perivitelline membrane (avians) and the zona pellucida (eutherian and marsupial mammals). These extracellular matrices perform similar functions with respect to establishing taxon-specific sperm–egg binding at fertilization and in protecting the embryo during early development. The physiology of mammalian fertilization has been intensely investigated in mice, in which relatively few sperm encounter the ovulated egg in the ampulla of the oviduct. Only motile, acrosome-intact sperm penetrate the cumulus oophorus and bind to the zona pellucida. This binding triggers the sperm acrosome reaction and acrosome-reacted sperm penetrate the zona matrix to bind and fuse with the vitelline (plasma) membrane of the egg. Following fertilization, peripheral cortical granules fuse with the vitelline membrane discharging their poorly characterized contents into the perivitelline space. This modifies the zona pellucida matrix such that sperm no longer bind or penetrate (Fig. 1).

This review will focus on recent molecular and genetic investigations into the role of the mouse and, to a lesser extent, human zona pellucida in fertilization. The former provides a well-developed model system with tractable genetics and the latter reflects an innate curiosity about the biology of our own species and relevant fertility issues.

Composition and structure of the mouse zona pellucida

The mouse zona pellucida is composed of three glycosylated proteins designated ZP1, ZP2 and ZP3. The primary amino acid sequences of ZP1 (623 amino acids), ZP2 (713 amino acids) and ZP3 (424 amino acids) were initially deduced from full-length cDNA (Ringuette et al. 1988, Liang et al. 1990, Epifano et al. 1995). Each zona protein is synthesized as a nascent polypeptide chain with an N-terminal signal sequence and a C-terminal transmembrane domain followed by a short cytoplasmic tail. The signal sequence is cleaved in the endoplasmic reticulum generating mature N-termini of ZP1 (q^{21}), where q represents pyroglutamic acid, ZP2 (Val^{15}) and ZP3 (q^{13})(Liang et al. 1990, Boja et al. 2003). As the three proteins traffic through the cell, they are extensively glycosylated and the resultant carbohydrate side chains account for roughly half of the mass of each protein (Nagdas et al. 1994, Easton et al. 2000). The occupancy of potential
glycosylation sites by carbohydrate side chains in native zonae pellucidae has been assessed by mass spectrometry (Boja et al. 2003). There are multiple asparagines that fall within a consensus sequence for N-linked glycosylation in ZP1 (4 sites), ZP2 (6 sites) and ZP3 (6 sites) and all, except for one in ZP3, are occupied by sugar side chains. In contrast, little or no O-glycosylation is detected on ZP2 and few O-glycans are detected on ZP3. For example, of the 58 serine and threonine residues in mature ZP3 no more than five appear to be glycosylated (Boja et al. 2003); they do not include Ser\textsuperscript{332} or Ser\textsuperscript{334} previously implicated as attachment sites for O-glycans involved in sperm binding (Chen et al. 1998).

It is likely that the zona proteins contain signals to direct their trafficking through the growing oocytes (Zhao et al. 2003) and facilitate their interactions with one another either through subassembly in cellular compartments or as they oligomerize within the extracellular matrix. The mechanisms by which the glycosylated ectodomains of the zona proteins are released from the anchoring transmembrane domain remain to be determined. Although the mouse zona proteins contain a proprotein convertase (RX(K/R)R) site N-terminal to their transmembrane domains, they remain associated with the plasma membrane (Rankin et al. 1996, Qi et al. 2002, Zhao et al. 2002) despite their obligatory passage through the trans-Golgi network wherein resides the endoprotease (Thompson 2002). The C-termini of mouse ZP1, ZP2 and ZP3 isolated from native zonae pellucidae end immediately upstream of a dibasic motif that is part of, but distinct from, the convertase cleavage site (Boja et al. 2003). The dibasic motif is conserved in all mammalian species examined to date and similarly positioned C-termini in quail, Xenopus and cow homologues of ZP3 indicate conserved mechanisms for C-terminal processing (Kubo et al. 1999, Sasanami et al. 2002, Yonezawa & Nakano 2003). However, the observation that mutation of the dibasic motif in ZP3 (RNRR → ANAA or → RNGE) does not preclude secretion or incorporation into the zona pellucida even in transgenic mice suggests that alternative cleavage sites are available (Kiefer & Saling 2002, Qi et al. 2002, Zhao et al. 2002).

Due to these post-translational modifications, mature mouse ZP1, ZP2 and ZP3 have apparent molecular masses of 180–200, 120–140 and 83 kDa respectively (Bleil & Wassarman 1980, Shimizu et al. 1983). Genetic data from mice lacking either ZP1, ZP2 or ZP3 indicate that a zona matrix can be formed with either ZP1 and ZP3, or ZP2 and ZP3, although the former matrix is quite thin and does not persist through oogenesis (Rankin et al. 1999, 2001). There is also experimental evidence suggesting that the well-conserved ‘zona domain’, formed by 260 amino acids with eight cysteine residues (Bork & Sander 1992), plays a role in polymerization of the zona proteins (Jovine et al. 2002). These data, and the observation that the disulfide linkages in the ZP1 and ZP2 zona domains appear to differ from those of ZP3 (Boja et al. 2003), are consistent with a model in which the zona filaments are composed of interspersed heterodimers of ZP1/ZP3 and ZP2/ZP3. The limiting amounts of ZP1 (least abundant of the three proteins) may account for the thinness of the ZP1/ZP3 zona matrix, but the ability of ZP1 to form inter-molecular disulfide bonds may allow it to provide structural stability disproportionate to its mass within the normal zona pellucida.

**Sperm binding to the zona pellucida**

Sperm bind and penetrate the zona pellucida of eggs but not two-cell embryos and credible models of sperm–egg recognition must account for this dichotomy (Fig. 2). Although post-fertilization modifications of ZP3 have been inferred, the only biochemical change documented in the zona matrix is cleavage of ZP2, after which the N- and C-terminal fragments remain intramolecularly disulfide bonded.

**Single-protein models**

Individual zona proteins have been implicated as sperm ‘receptors’ in different species, but there is no loss of

Figure 1 Mouse fertilization. A relatively few number of capacitated, acrosome-intact sperm encounter ovulated eggs (arrested in metaphase II) in the ampulla of the oviduct (left panel). Sperm binding to the zona pellucida induces the acrosome reaction and usually a single sperm penetrates the zona pellucida to fuse with the plasma membrane, after which the entire sperm enters into the cytoplasm (middle panel). Fertilization triggers cortical granule migration to the cell surface and its exocytosis that occurs within minutes modifies the zona pellucida (red → yellow) so that sperm no longer bind and those in the process of penetration progress no further. This results in monospermic fertilization with single male and female pronuclei in the one-cell zygote (right panel).
protein to account for the post-fertilization absence of sperm binding to the zona pellucida. More recent genetic studies in mice also have not been supportive of single-protein models. Mice lacking ZP1 form a zona pellucida composed of ZP2 and ZP3 which, although structurally flawed, continues to support sperm binding and Zp1 null mice are fertile (Rankin et al. 1999). Thus, if sperm binding is predicated on a single zona protein, it is unlikely to be ZP1. Normally human sperm will not bind to mouse zonae pellucidae (Bedford 1977) and it was reasoned that if sperm bind to either ZP2 or ZP3, the presence of the cognate human zona proteins would alter the specificity of binding. Therefore, endogenous mouse ZP2 or ZP3 were replaced with human homologues to establish huZP2 or huZP3 rescue mice. Each line is fertile validating the integrity of the human–mouse chimeric zona pellucida, but neither the presence of human ZP2 nor human ZP3 is sufficient to support human sperm binding in vitro (Rankin et al. 1998, 2003). Together these results do not support the role of a single protein in taxon-specific sperm binding and alternative explanations have been sought.

**Single-glycan models**

Existing models that postulate specific O- or N-linked glycans as sperm ‘receptors’ (Florman & Wassarman 1985, Miller et al. 1992, Tulsiyan et al. 1997) invoke their release by cortical granule glycosidases to account for the absence of post-fertilization sperm binding (e.g. Miller et al. 1993). The well-developed ZP3 glycan model of mouse sperm–zona recognition proposes that sperm bind to O-glycans linked to ZP3 and that these saccharides are removed following fertilization. The genesis of this model was the experimental observation that SDS-PAGE-purified ZP3 from eggs, but not two-cell embryos, inhibited sperm binding to eggs and induced the acrosome reaction in a dose-dependent manner. Similarly isolated ZP1 and ZP2 had neither of these activities and the sperm-binding, but not the acrosome-inducing, activity persisted with ZP3 glycopeptides or biochemically isolated ZP3 O-glycans. Subsequent experiments have led to progressive refinements of the model which, in its current iteration, ascribes sperm binding activity to O-linked oligosaccharide side chains attached to Ser332 and Ser334 of ZP3 (for review see Wassarman 2002).

Both terminal α-1,3 galactose and N-acetylglucosamine have been implicated as the ‘sperm receptor’ within this model. However, genetically engineered mice that lack the galactosyl transferase required for the addition of α-1,3 galactose to ZP3 are fertile (Thall et al. 1995, Liu et al. 1997), as are male mice lacking the sperm surface β-1,4 galactosyl transferase isoform thought to bind N-acetylglucosamine residues on ZP3 (Asano et al. 1997, Lu & Shur 1997). Neither terminal O-linked N-acetylglucosamines (Easton et al. 2000) nor O-glycans on Ser332 or Ser334 of ZP3 (Boja et al. 2003) are detected by sensitive mass spectrometry analysis of native zona pellucidae. Moreover, mice in which Ser332 or Ser334 were mutated to preclude occupancy by O-linked sugars are fully fertile in vivo (Liu et al. 1995), although the definitive assessment of their reproductive fitness in the Zp3 null background has not been reported.

The further observation that sperm can bind to two-cell embryos despite cortical granule exocytosis (provided that ZP2 remains intact) is difficult to reconcile with ZP glycan models (Rankin et al. 2003). In particular, it is hard to envision a single carbohydrate side chain or terminal sugar that would remain accessible for sperm binding and yet be inaccessible for cleavage by a cortical granule...
glycosidase. Thus, these recent genetic data do not appear consistent with sperm binding to a single O- or N-glycan, the release of which is dependent on glycosidase(s) discharged during post-fertilization cortical granule exocytosis.

**Supramolecular models**

Because these genetic data do not support models in which sperm binding is dependent on individual mouse zona proteins or a particular glycan that is cleaved off following fertilization, attention has been drawn to the supramolecular structure of the zona matrix (Fig. 3). These more recent formulations have been guided by the striking persistence of sperm binding to two-cell embryos in huZP2 and huZP2/ZP3 rescue mice which correlates with uncleaved huZP2 despite cortical granule exocytosis (Rankin et al. 2003). This has led to a model in which the zona pellucida composed, *ad minimus*, of ZP2 and ZP3 forms a three-dimensional matrix around ovulated eggs to which sperm will bind. Normally, after fertilization the cortical granules exocytose a protease that cleaves ZP2 (Barros & Yanagimachi 1971, Gwatkin et al. 1973, Wolf & Hamada 1977). This cleavage modifies the supramolecular structure of the zona matrix rendering it unable to support sperm binding. Although not precluded, loss of carbohydrate or protein from the zona matrix is not required in this formulation. Normally human ZP2 is cleaved following fertilization (Bauskin et al. 1999) and why it remains uncleaved in the mouse–human chimeric zonae is yet to be determined. Although there is some electron microscopic evidence that a structural change does indeed occur in the zona matrix following fertilization (Baranska et al. 1975, Jackowski & Dumont 1979, Funahashi et al. 2001), more detailed studies of the zona matrix are required to fully understand the molecular basis of sperm binding to ovulated eggs but not two-cell embryos.

**Taxon-specific sperm binding**

Speciation is well maintained among mammals by premating determinants (habitat, mate discrimination, physiognomy) and the need for taxon specificity during internal fertilization is not compelling. Nevertheless there is variation among mammals and while mouse sperm are promiscuous in egg recognition, human sperm are quite fastidious and will not bind to mouse eggs (Bedford 1977). ZP2 and ZP3 have been implicated as ‘sperm receptors’ and the human and mouse proteins are respectively 61 and 67% identical at the amino acid level (Rankin & Dean 2000). Unexpectedly, human sperm do not bind *in vitro* to ‘humanized’ zona pellucidae containing human ZP2 or human ZP3 or both (Rankin et al. 1998b). Although the absence of a human-specific O- or N-glycan was considered as a possible explanation, the two human proteins expressed in mouse eggs are post-translationally modified to a similar, if not identical, extent as native human ZP2 and ZP3. Furthermore, the role of specific O- and N-glycans in mediating sperm–egg recognition is not consistent with the post-fertilization persistence of sperm binding in humanized zonae.

As noted above, the continued fertility of Zp1 null mice suggests that ZP1 is not required for sperm binding and fertilization. However, it remains possible that the protein enforces structural constraints in the zona matrix that are critical for sperm recognition in a taxon-specific manner. Consistent with this formulation, mouse ZP1 (623 amino acids) and the third human protein (ZPB, 540 amino acids) are the least conserved, sharing only 42% amino acid identity. If these differences are critical in determining the

![Figure 3 Chimeric human–mouse zonae pellucidae. Using transgenesis, mouse lines have been established in which human ZP2 (huZP2) replaces endogenous mouse ZP2 (moZP2). Ovulated eggs obtained from these mice can be fertilized *in vitro* (left panel), but despite cortical granule exocytosis, sperm continue to bind to two-cell embryos (middle panel). Normally ZP2 is cleaved after fertilization (red → yellow), but huZP2 remains intact in the chimeric zonae (red). This suggests that uncleaved ZP2 maintains zona matrix in a state permissive to sperm binding and that the normal cleavage of ZP2 renders the matrix unable to support sperm binding. Mice in which huZP2 by itself or in conjunction with huZP3 replaces endogenous mouse proteins are fertile *in vivo* (right panel), although they have decreased ovulation and fecundity.](https://www.reproduction-online.org)
supramolecular structure of the zona matrix, transgenic mice in which human ZPB replaces endogenous mouse ZP1 may alter the specificity. Alternatively, sperm binding to humanized zonae may require that all three zona glycoproteins come from the same species (‘triple human rescue’), or simply that human ZP2 and human ZP3 be expressed on a mouse ZP1 null background.

It is also possible that humans possess an additional zona pellucida protein. An analysis of data compiled from the human genome project identified a potential human ZP1 gene that encodes a 638 amino acid protein similar in size and more homologous to mouse ZP1 (623 amino acids, 67% identity) than to human ZPB (540 amino acids, 42% identity) (Hughes & Barratt 1999). However, as yet neither oocyte nor ovarian expression has been reported and so this remains but an intriguing possibility.

**Post-fertilization block to polyspermy**

Although a few supernumerary sperm are normally observed in the perivitelline space after fertilization, additional sperm do not accumulate (Sato 1979). This ‘zona block’ appears to involve two distinct phases: first, prevention of sperm binding and then prevention of penetration through the zona matrix (Fig. 1). In rodents, the block to sperm binding can be mimicked by treatment of ovulated eggs with cortical granule exudates (Barros & Yanagimachi 1971) and recent genetic studies indicate that, irrespective of fertilization, it is the cleavage status of ZP2 that determines whether sperm bind to the zona pellucida (Rankin et al. 2003). The zona block to penetration has been difficult to study separately from mechanisms that affect sperm binding (i.e. if sperm don’t bind, they won’t penetrate), although historically the block to penetration has been associated with the post-fertilization cleavage of ZP2 (Moller & Wassarman 1989).

The fertility of huZP2 and huZP2/ZP3 rescue mice infers monospermic fertilization and an effective zona block to sperm penetration. This has been confirmed in vitro where, despite persistence of sperm binding to early embryos, excessive supernumerary sperm are not observed within the confines of the zona pellucida. Thus, the block to penetration does not appear dependent on cleavage of mouse ZP2 (Rankin et al. 2003). Earlier investigators in mouse and other vertebrates have implicated cortical granule exocytosis in the block to zona penetration. The cortical granule contents presumably diffuse into the zona pellucida and prevent sperm penetration either by enzymatically or mechanically modifying the zona matrix (Dandekar & Talbot 1992, Quill & Hedrick 1996, Green 1997). The mechanism by which this occurs in mice remains to be determined.

**Conclusions**

Impressive advances have been made over the last several decades in understanding mammalian fertilization. In large part this has been driven by revolutions in molecular biology, transgenesis and comparative genomics. Although it seems likely that many of the molecular mechanisms will be conserved among mammalian taxa, there may be subtle differences. In the past, investigations have focused on sperm–egg recognition mediated by the zona pellucida and have sought to identify a single zona protein or a single carbohydrate residue as a ‘receptor’ to which sperm bind. In these models, following fertilization the ‘sperm receptor’ is released from the zona pellucida and accounts for the post-fertilization absence of sperm binding. Recent genetic data on which this review has focused offer an alternative model based on the supramolecular structure of the zona pellucida. In this formulation, the zona pellucida surrounding ovulated eggs forms a matrix to which acrosome-intact sperm bind. Following fertilization and cortical granule exocytosis, the cleavage of ZP2 modifies the three-dimensional structure of the zona matrix so that sperm can no longer bind. Although not precluded, this process need not result in the loss of zona components. By mechanisms apparently independent of ZP2 cleavage, the exocytosed cortical granule contents modify the zona pellucida matrix to prevent penetration by additional sperm.

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