

Sperm–Zona Pellucida Interaction: Molecular Mechanisms and the Potential for Contraceptive Intervention

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Abstract At the moment of insemination, millions of mammalian sperm cells are released into the female reproductive tract with the single goal of finding the oocyte. The spermatozoa subsequently ignore the thousands of cells they make contact with during their journey to the site of fertilization, until they reach the surface of the oocyte. At this point, they bind tenaciously to the acellular coat, known as the zona pellucida, which surrounds the oocyte and orchestrate a cascade of cellular interactions that culminate in fertilization. These exquisitely cell- and species- specific recognition events are among the most strategically important cellular interactions in biology. Understanding the cellular and molecular mechanisms that underpin

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them has implications for the etiology of human infertility and the development of novel targets for fertility regulation. Herein we describe our current understanding of the molecular basis of successful sperm–zona pellucida binding.

Keywords Capacitation · Fertilization · Spermatozoa

1 Introduction

The continuation of all mammalian species relies on the cellular interactions that occur between gametes during the process of fertilization. These remarkable cell- and species-specific interactions are initiated by recognition and binding of a spermatozoon to the zona pellucida (ZP), a thick extracellular matrix that surrounds and protects the ovulated oocyte. Understanding the basic biology of this interaction has profound implications for the diagnosis of human infertility and the development of novel targets for fertility regulation. Accordingly, considerable research effort has been devoted to investigating the molecular mechanisms that underpin sperm–oocyte interaction. Nonetheless, this fundamental interaction remains poorly understood and is the subject of considerable controversy.

Studies of sperm–zona pellucida adhesion conducted during the last 60 years have led to the advancement of a widely accepted paradigm that sperm–ZP interaction is mediated by a single sperm receptor that engages with a complementary ligand within the ZP. While such a model holds obvious appeal, it fails to account for the fact that targeting of individual sperm proteins through inhibition studies (e.g., competitive substrates and mono-specific antibodies) and/or genetic deletion has failed to elicit the anticipated block to sperm–ZP interaction. Against this background we have introduced a novel hypothesis which states that sperm–ZP interaction requires the coordinated action of several sperm proteins, each of which contributes to the high affinity and specificity of this fundamental cellular interaction. Furthermore, we have also suggested that these discrete zona recognition proteins are assembled into a multimeric receptor complex during sperm capacitation. Throughout this review, we have chosen to focus on the mouse model as it represents the most widely studied of all laboratory animals with respect to mammalian fertilization. However, the authors encourage caution in extrapolating these data for cross-species comparison.

2 Sperm–Zona Pellucida Interaction

2.1 *The Zona Pellucida*

The zona pellucida (ZP) is synthesized during oogenesis and is located between the oocyte and the innermost layer of granulosa cells (Wassarman and Albertini 1994). The mature ZP is a porous matrix whose functions include the mediation of

species-specificity in gamete interaction (Vieira and Miller 2006), prevention of polyspermy, and protection of the developing embryo prior to implantation (McLeskey et al. 1998). The importance of these functions has been underscored by studies involving the targeted deletion of the genes encoding ZP proteins (Rankin and Dean 2000). Such studies have shown that the ZP is essential in maintaining the physiological status of the oocyte and in regulating successful growth and development of the embryo. Furthermore, it has been demonstrated that oocytes failing to correctly translate and assemble a ZP are unable to be fertilized (Liu et al. 1996; Rankin et al. 1996). Indeed, this structure represents the first barrier that mammalian sperm must encounter and breach to achieve fertilization.

In the mouse, the ZP is assembled as a trimeric protein matrix composed of long ZP2 and ZP3 heterodimer filaments that are cross-linked by homodimers of the third zona protein, ZP1 (Bleil and Wassarman 1980b; Greve and Wassarman 1985; Wassarman and Mortillo 1991) in a molar ratio of 4:4:1, respectively (Green 1997) (Fig. 1). The three mouse ZP proteins were initially characterized by Bleil and Wassarman (1980a, b, c) and have subsequently been shown to be encoded by single-copy genes located on chromosomes 19, 7, and 5 (Epifano et al. 1995). Determination of the primary structure of the ZP proteins revealed considerable divergence between their predicted molecular weight and that determined experimentally via SDS-PAGE (ZP1, ~200 kDa; ZP2, ~120 kDa; and ZP3, ~83 kDa). Such differences are accounted for by dramatic posttranslational modification of the mature proteins, primarily in the form of glycosylation (Ringuette et al. 1986; Kinloch et al. 1988; Liang et al. 1990; Epifano et al. 1995), a feature which appears critical for their biological activity. The oligosaccharides are in turn modified by sulfation, sialylation, and the addition/removal of other moieties (Liu et al. 1997).

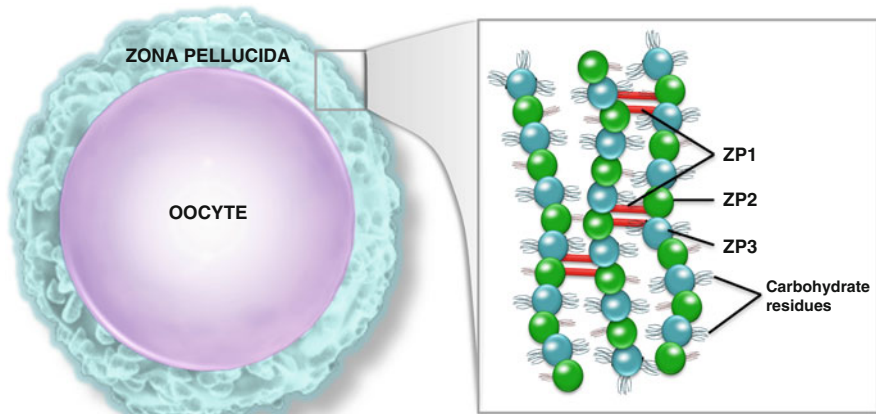


Fig. 1 *The mouse zona pellucida.* The ovulated oocyte is surrounded by the ZP, an acellular matrix whose functions include the mediation of species-specificity in gamete interaction, prevention of polyspermy, and protection of the developing embryo prior to implantation. The mouse zona pellucida is a fibrillar structure, the major strands of which are composed of repeating dimers of ZP2 and ZP3 glycoproteins. These strands are crosslinked by ZP1 to form a mesh-like network

As well as being essential structural components of the zona pellucida, ZP2 and ZP3 possess specific functions during the sequence of sperm–oocyte interactions that culminate in fertilization. The balance of evidence indicates that mouse ZP3 functions as both the primary sperm receptor, preferentially binding the plasma membrane region overlying the acrosome of acrosome-intact sperm, and as an inducer of the acrosome reaction following recognition of the zona matrix (Bleil and Wassarman 1986; Vazquez et al. 1989). For instance, it has been demonstrated that solubilized ZP3 is able to competitively inhibit sperm–ZP binding, whereas ZP1 and ZP2 do not elicit a similar response (Bleil and Wassarman 1980a). Similarly, female mice bearing a null mutation for ZP3 are infertile (Liu et al. 1996). It is noteworthy, however, that ZP3 is not uniquely responsible for facilitating sperm interaction in all mammalian species. In the pig for instance, sperm-binding activity resides in a heterodimer formed between the ZP1 and the ZP3 orthologues (Yurewicz et al. 1998). Similarly, the ZP1 orthologue, ZPB, has been shown to play a major role in sperm binding to the bovine zona (Yonezawa et al. 2001), while in humans and the bonnet monkey there is also compelling evidence that a fourth zona glycoprotein, ZP4, which is thought to be dysfunctional in the mouse (Lefievre et al. 2004), participates in primary sperm adhesion (and the induction of acrosomal exocytosis) (Gupta et al. 2007). The biological significance of this interspecies complexity in ZP structure and function is presently unknown. Nonetheless, given that the mouse remains the most widely studied model for understanding sperm–ZP interaction, this species will serve as the focus for the following discussion.

The mouse ZP3 glycoprotein comprises a number of domains including an N-terminal signal sequence, a large ZP domain, a consensus furin cleavage site (CFCS), and a hydrophobic transmembrane region located near the C-terminus. The ZP domain is in fact common to all ZP proteins and consists of a 260 amino acid sequence with eight conserved cysteine residues. This domain is believed to be responsible for the polymerization of the ZP proteins into the extensive lattice-like network that enables it to surround the oocyte (Jovine et al. 2002). Mouse ZP3 is initially synthesized as a 424 amino acid polypeptide, but is subject to dramatic posttranslational modification resulting in the addition of complex N-(asparagine) and O-linked (serine/threonine) carbohydrates. The sperm-binding domain of the ZP3 glycoprotein has been mapped to the C-terminus of the protein and encompasses both the ZP and CFCS domains (Litscher et al. 2009). This region of the polypeptide is commonly referred to as the sperm combining site and contains O-linked sugar residues that putatively interact with complementary receptors on the surface of acrosome intact spermatozoa (Wassarman et al. 2004). However, as discussed below, this model of sperm–ZP interaction is not universally accepted.

2.1.1 The Role of O-linked ZP3 Sugars in Mouse Sperm–ZP Interaction

Prevailing evidence indicates that primary sperm–oocyte interaction is mediated by binding between ZP3 carbohydrates (Gwatkin et al. 1977; Hoodbhoy and Dean 2004) and complementary lectin-like proteins located on the surface of the sperm

(see Sect. 2.2 and Table 1). The most widely accepted model of this interaction emphasizes the importance of O-linked carbohydrate moieties (Florman and Wassarman 1985; Litscher et al. 1995; Wassarman et al. 1999). For instance, it has been demonstrated that small glycopeptides derived from mouse ZP3 retain full sperm receptor activity (Florman et al. 1984). Conversely, the enzymatic and chemical removal or modification of O-linked oligosaccharides from ZP3 abolishes its sperm receptor activity whereas the removal of N-linked oligosaccharides elicits only negligible effects (Florman and Wassarman 1985). The importance of O-linked glycans has been further advanced by the demonstration that genetically engineered chimeric mouse oocytes expressing human ZP3 acquire the same O-linked glycans as mouse ZP3 and bind mouse, rather than human, spermatozoa (Rankin et al. 1996; Hoodbhoy and Dean 2004). Furthermore, the results of targeted mutagenesis studies indicate that the key O-linked carbohydrates responsible for sperm-binding activity most likely reside within the C-terminal portion of the ZP3 polypeptide chain (Kinloch et al. 1995).

However, while it is widely accepted that the principal bioactive component of ZP3 is associated with its O-linked carbohydrate moieties, the relative importance of the different oligosaccharide ligand(s) remains to be unequivocally established (Easton et al. 2000; Diekman 2003). This situation is due in part to the complexity of O-linked glycans, with recent mass spectrometry analysis of mouse ZP3 glycosylation revealing that the predominant core type 2 sequences are terminated with sialic acid, lacNAc (Gal β 1-4GlcNAc), lacdiNAc (Gal-NAc β 1-4GlcNAc), Gal α 1-3Gal, and NeuAc α 2-3[GalNAc β 1-4]Gal β 1-4 (Sda antigen) (Dell et al. 2003). Early studies suggested that galactose, located in an α -linkage at the nonreducing terminus of O-linked oligosaccharides served as a critical determinant of sperm binding to ZP3 (Florman and Wassarman 1985; Bleil and Wassarman 1988; Litscher et al. 1995). However, such claims have since been refuted (Nagdas et al. 1994; Thall et al. 1995) in favor of terminal β -linked galactose (Yonezawa et al. 2005) in addition to *N*-acetylglucosamine (Miller et al. 1992), mannose (Tulsiani et al. 1989; Cornwall et al. 1991), *N*-acetylgalactosamine, and fucose residues (Johnston et al. 1998; Kerr et al. 2004), each of which have been demonstrated to inhibit sperm-zona binding (reviewed in Benoff 1997). Arguments against the involvement of α -linked galactose residues include the demonstration that sperm-ZP interaction is inhibited by pretreatment of oocytes with β -galactosidase but not α -galactosidase (Mori et al. 1997). Furthermore, female transgenic mice bearing a null mutation for α 1 \rightarrow 3 galactosyltransferase (and therefore terminal Gal α 1 \rightarrow 3Gal residues) produce oocytes that display normal sperm-binding characteristics (Thall et al. 1995). These data are further supported by evidence from a novel heterologous cell-adhesion assay between mouse spermatozoa and rabbit erythrocytes. The precocious binding of these two cell types appears to be attributed to the presence of multiple branches of Gal α 1 \rightarrow 3Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 6 linked to a linear polyglucosamine backbone in the erythrocytes (Clark et al. 1996; Clark and Dell 2006; Sutton-Smith et al. 2007). However, pretreatment of the erythrocytes with α -galactosidase fails to elicit the anticipated reduction in sperm adhesion (Clark and Dell 2006), thereby suggesting sperm

Table 1 Putative sperm-ZP receptor candidates

Name (synonyms)	Species	Evidence	References			
α -D-mannosidase (MAN2B2)	Mouse	<ul style="list-style-type: none"> Integral plasma membrane protein that may participate in sperm-ZP interaction by binding to mannose-containing ZP saccharides Treatment of sperm with either D-mannose or an antibody raised against the protein elicit a dose-dependent inhibition of sperm-ZP binding Acquired from epididymal fluid via interaction with SGG and localizes to the membrane overlying the acrosome A dose-dependent decrease in sperm-ZP binding is observed following pretreatment of sperm with ARSA antibodies ARSA null males are fertile Transmembrane protein located on dorsal surface of the anterior sperm head overlying the intact acrosome Acts as a receptor for terminal GlcNAc residues on ZP3 ZP3-induced GalTase aggregation triggers a pertussis toxin-sensitive G-protein cascade leading to induction of acrosomal exocytosis Transgenic mice overexpressing GalTase are hypersensitive to ZP3 and undergo precocious acrosome reactions Sperm from mice bearing targeted deletions in GalTase are unable to bind ZP3 nor undergo ZP3-dependent acrosomal exocytosis However, GalTase-null sperm retain the ability to bind to the egg coat 	(Tuisiani et al. 1989, 1993; Cornwall et al. 1991; Pereira et al. 1998; Yoshida-Komiya et al. 1999; Akama et al. 2002)			
	Rat					
	Hamster Human					
Arylsulfatase A (AS-A; ARSA)	Mouse	<ul style="list-style-type: none"> Localized to postacrosomal region of sperm head Immunization of mice with recombinant FA-1 antigen induces a strong immunoprotective response Anti-FA-1 antibodies have been implicated in immune infertility in humans No recorded knockout mouse Two isoforms (Pi and Mu) localized to periphery of plasma membrane over anterior head, postacrosomal region and principal piece 	(Hess et al. 1996; Tantibhedhyangkul et al. 2002; Weerachayanukul et al. 2003)			
	Human					
	Boar					
β -1,4-galactosyltransferase (GalTase; GALT; B4GALT1)	Mouse	<ul style="list-style-type: none"> Localized to postacrosomal region of sperm head Immunization of mice with recombinant FA-1 antigen induces a strong immunoprotective response Anti-FA-1 antibodies have been implicated in immune infertility in humans No recorded knockout mouse Two isoforms (Pi and Mu) localized to periphery of plasma membrane over anterior head, postacrosomal region and principal piece 	(Shur and Hall 1982; Lopez and Shur 1987; Lu and Shur 1997; Shi et al. 2001)			
	Rat					
	Human					
	Guinea pig					
	Rabbit					
	Bull					
	Boar					
	Stallion					
	Fertilization antigen 1 (FA1)			Mouse	<ul style="list-style-type: none"> Localized to postacrosomal region of sperm head Immunization of mice with recombinant FA-1 antigen induces a strong immunoprotective response Anti-FA-1 antibodies have been implicated in immune infertility in humans No recorded knockout mouse Two isoforms (Pi and Mu) localized to periphery of plasma membrane over anterior head, postacrosomal region and principal piece 	(Naz et al. 1984, 1992; Coonrod et al. 1994; Naz and Zhu 1998; Menge et al. 1999)
				Human		
Bull						
Glutathione S-transferase (GST)	Mouse	<ul style="list-style-type: none"> Localized to postacrosomal region of sperm head Immunization of mice with recombinant FA-1 antigen induces a strong immunoprotective response Anti-FA-1 antibodies have been implicated in immune infertility in humans No recorded knockout mouse Two isoforms (Pi and Mu) localized to periphery of plasma membrane over anterior head, postacrosomal region and principal piece 	(Shaha et al. 1988; Aravinda et al. 1995; Gopalakrishnan et al. 1998; Hemachand et al. 2002)			
	Rat					

Human Goat	<ul style="list-style-type: none"> • Selective inhibition of Pi², but not Mu, isoform with specific antisera leads to a reduction in fertilization rate • Both isoforms bind to ZP3 • An EGF repeat and discoidin domain protein that coats sperm (plasma membrane overlying the acrosome) within the epididymis • Binds specifically to the ZP of unfertilized but not fertilized eggs • Recombinant MFGE8 and anti-MFGE8 antibodies competitively inhibit sperm-egg binding • MFGE8 null males are subfertile and their sperm are unable to bind to the ZP <i>in vitro</i> 	(Ensslin et al. 1995, 1998; Ensslin and Shur 2003)
Milk fat globule-EGF factor 8 (MFGE8; P47; SED1)	<ul style="list-style-type: none"> • Localizes to acrosome and inner acrosomal membrane from where it is thought to mediate secondary binding to ZP2 following acrosomal exocytosis • Binding to ZP is nonenzymatic and thought to involve recognition of polysulphate groups on ZP glycoproteins • Acrosin null males are fertile but show compromised ZP penetration 	(Urch and Patel 1991; Baba et al. 1994; Howes et al. 2001; Howes and Jones 2002)
Proacrosin (acrosin)	<ul style="list-style-type: none"> • Widely conserved sperm surface protein • Localized to plasma membrane over anterior head of mouse sperm • Possesses hyaluronidase activity that assists in the digestion of cumulus cells • Relocalizes to inner acrosomal membrane following acrosome reaction where it is putatively involved in secondary ZP binding 	(Lin et al. 1994; Hunnicutt et al. 1996; Myles and Primakoff 1997; Baba et al. 2002; Morales et al. 2004)
Sperm adhesion molecule 1 (SPAM1; PH-20)	<ul style="list-style-type: none"> • SPAM1 null males are fertile but their sperm are less efficient in dispersal of cumulus cells • SPAM17 is a conserved, highly antigenic protein variously localized to acrosome and flagellar fibrous sheath • Has been implicated in regulation of sperm maturation, capacitation, acrosome reaction and ZP binding • Shown to bind specific carbohydrate components (mannose) of the ZP 	(Yamasaki et al. 1995; Grizzi et al. 2003; Chiriva-Internati et al. 2009)
All mammals	<ul style="list-style-type: none"> • Sperm autoantigenic protein 17 (SPA17, SP17) 	(Yamasaki et al. 1995; Grizzi et al. 2003; Chiriva-Internati et al. 2009)
Mouse Rabbit Human Primates	<ul style="list-style-type: none"> • Sperm autoantigenic protein 17 (SPA17, SP17) 	(Yamasaki et al. 1995; Grizzi et al. 2003; Chiriva-Internati et al. 2009)

(continued)

Table 1 (continued)

Name (synonyms)	Species	Evidence	References
Spermadhesins (AWN; AQN-1; AQN-3)	Boar Stallion Bull	<ul style="list-style-type: none"> • Spermadhesin represents a major component of seminal plasma and is deposited over the sperm head via interaction with plasma membrane phospholipids • Multifunctional lectins able to bind to carbohydrates, sulfated glycosaminoglycans, phospholipids and protease inhibitors • May therefore participate in several sequential steps of the fertilization process 	(Sanz et al. 1993; Sinowitz et al. 1995; Topfer-Petersen et al. 1998; Petrunkina et al. 2000)
Sulfogalactosylglycerolipid (SGG)	Mouse Rat Human Boar	<ul style="list-style-type: none"> • Localizes to plasma membrane overlying acrosome and postacrosomal regions of mouse and human spermatozoa. Restricted to anterior head plasma membrane of boar sperm • SGG is a major sperm sulfoglycolipid that putatively facilitates the uptake of sulfolipid-immobilizing protein-1 (SLIPT) and ARSA • Following capacitation, SGG is predominantly found in membrane rafts, microdomains that possess ZP affinity • Pretreatment of sperm with monovalent anti-SGG Fab fragments markedly inhibits sperm binding to the ZP 	(Komblatt 1979; Tamphaichitr et al. 1990, 1993; White et al. 2000; Weerachayanukul et al. 2001; Bou Khalil et al. 2006)
Zonadhesin (ZAN)	Mouse Hamster Rabbit Boar Bull Horse Primates	<ul style="list-style-type: none"> • ZAN localizes to the apical head overlying the acrosome following spermatogenesis and epididymal maturation • The protein displays testis-specific expression • ZAN features a mosaic protein architecture with several domains (MAM, mucin, D-, and EGF) that putatively enable the protein to participate in multiple cell adhesion processes including ZP binding 	(Hardy and Garbers 1994, 1995; Hickox et al. 2001; Bi et al. 2003; Olson et al. 2004; Gasper and Swanson 2006; Herlyn and Zischler 2008)

Zona pellucida binding proteins 1 & 2 (ZPBPI; Sp38; IAM38; ZPBPI2)	<p>Mouse Human Boar</p>	<ul style="list-style-type: none"> ● Both ZPBPI and its paralog, ZPBPI2, localize to the sperm acrosome and are expressed exclusively in the testes of mice and humans ● Both proteins have been implicated in secondary ZP binding ● Preincubation of boar spermatozoa with anti-ZPBPI antibodies block in vitro fertilization ● ZPBPI null males produce sperm with abnormal head morphology and no forward motility and are consequently sterile ● ZPBPI2 null males produce dysmorphic sperm with reduced ability to penetrate zona pellucida ● ZPBPI2 is an intraacrosomal protein but may be presented to surface of the apical region of the sperm head region following capacitation ● Evidence exists that ZPBPI2 may be involved in either primary and/or secondary ZP interactions ● Oocytes pretreated with recombinant ZPBPI2 show a dose dependant decrease in sperm affinity ● No recorded knockout mouse 	<p>(Mori et al. 1993, 1995; Yu et al. 2006; Lin et al. 2007)</p>
ZP3 receptor protein (ZP3R; SP56; AM67)	<p>Mouse Rat Guinea pig</p>	<ul style="list-style-type: none"> ● ZP3R is an intraacrosomal protein but may be presented to surface of the apical region of the sperm head region following capacitation ● Evidence exists that ZP3R may be involved in either primary and/or secondary ZP interactions ● Oocytes pretreated with recombinant ZP3R show a dose dependant decrease in sperm affinity ● No recorded knockout mouse 	<p>(Bleil and Wassarman 1990; Cheng et al. 1994; Foster et al. 1997; Cohen and Wassarman 2001; Kim et al. 2001a, b; Kim and Gerton 2003; Buffone et al. 2008b)</p>

instead interact with β 1 \rightarrow 4-linked glycans. These results indicate that sperm can recognize terminal Gal β 1 \rightarrow 4GlcNAc sugars (Mori et al. 1997), which interestingly, are essentially the same structures in both ZP3 and ZP2 (Noguchi and Nakano 1993).

2.1.2 The Role of N-Linked ZP3 Sugars in Sperm–ZP Interaction

In addition to the classes of O-linked oligosaccharides described above, murine ZP3 is also known to be furnished with both high mannose and complex-type N-glycans (Easton et al. 2000). The predominant high mannose-type glycan is composed of Man5GlcNAc2, whereas the array of biantennary, triantennary, and tetraantennary complex-type N-glycans have been shown to be terminated with the following antennae: Gal β 1–4GlcNAc, NeuAc α 2–3Gal β 1–4GlcNAc, NeuGc α 2–3Gal β 1–4GlcNAc, the Sda antigen, and terminal GlcNAc (Easton et al. 2000). Interestingly, with the exception of the latter sugar, these N-glycan sequences resemble those that terminate the β 1–6-linked branches of ZP3 O-glycans. Such findings raise the prospect that N-linked glycans may also contribute to sperm adhesion. Indeed, in species such as the pig, N-linked and not the O-linked carbohydrates appear to mediate sperm–ZP interaction (Yonezawa et al. 1995; Nakano et al. 1996). It may therefore be argued that carbohydrate moieties of the ZP glycoproteins may be underpinning the species-specificity associated with sperm–ZP interaction.

2.1.3 Carbohydrate-Independent Models of Sperm–ZP Interaction

Notwithstanding the compelling evidence in favor of carbohydrate residues as the main determinant in mediating sperm–ZP interaction, the production of transgenic mice bearing null mutations for key glycosyltransferases has also raised some doubt regarding the overall necessity of ZP carbohydrates for binding sperm. For instance, female mice singly deficient in any one of the three known glycosyltransferases that generate core 2 O-glycans (C2GnT1, C2GnT2, and C2GnT3), and therefore many of the O-glycans normally found in the zona are fertile (Ellies et al. 1998; Stone et al. 2009). Remarkably, elimination of all three C2GnTs is also permissive of fertility (Ellies et al. 1998). Similarly, mice that do not possess MGAT-I, the enzyme that initiates complex and hybrid-type glycan synthesis, produce oocytes that retain the ability to be fertilized (Shi et al. 2004).

Among the carbohydrate-independent models that have been proposed, recent analyses conducted by Tanphaichitr and colleagues have raised the interesting prospect that the sulfation of ZP glycans may play a key role in sperm adhesion (Tanphaichitr et al. 2007). Specifically, it has been postulated that sulfated sugar residues of ZP3 serve as a ligand for a sulfatase enzyme, arylsulfatase-A (ARSA),

that is added to the sperm surface during post-testicular maturation and becomes annexed within the apical region of the sperm head following capacitation (Tanphaichitr et al. 1993; White et al. 2000; Weerachatanukul et al. 2001, 2003; Carmona et al. 2002; Tantibhedhyangkul et al. 2002) (see Table 1). Support for this model rests with the demonstration that the components of the zona pellucida (Prasad et al. 2000), as well as the sperm surface (Murray et al. 1980), are highly sulfated in nature. Furthermore, it has been shown that a range of synthetic sulfated substrates (including arylsulfates, sulfated monosaccharides, and ascorbate 2-sulfate) are capable of competitively inhibiting the fertilization of hamster oocytes *in vitro* at the level of sperm–zona binding (Ahuja and Gilbert 1985). In addition, the exposure of spermatozoa to exogenous enzymes capable of desulfating biological macromolecules (such as cerebroside, glycosaminoglycans and glycoproteins), significantly inhibits their zona binding affinity (Ahuja and Gilbert 1985). We have recently observed comparable levels of inhibition following treatment of mouse spermatozoa and oocytes with a similar range of reagents (Nixon et al., unpublished).

At present, the nature of sulfated zona binding sites on spermatozoa remains to be fully elucidated; however, these findings take on added significance in light of the recent report that male mice bearing a targeted deletion of the gene for protein-tyrosine sulfotransferase 2 (TPST2) are infertile (Borghesi et al. 2006). TPST2 is one of two closely related isoenzymes that mediate the tyrosine O-sulfation of a myriad of substrates such as adhesion molecules, G-protein-coupled receptors, coagulation factors, serpins, extracellular matrix proteins, and hormones, in both mice and humans (Beisswanger et al. 1998). TPST2 null mice have normal spermatogenesis and produce normal numbers of epididymal sperm that appear indistinct from their wild-type counterparts in terms of their morphology, motility, ability to capacitate *in vitro*, and undergo acrosome exocytosis in response to an agonist (Borghesi et al. 2006). However, they are severely defective in terms of their ability to fertilize ZP-intact eggs. The substrates for tyrosine O-sulfation in spermatozoa await further investigation.

Interestingly, in addition to the carbohydrate and sulfate residues that adorn the mature ZP proteins, a small number of studies have also suggested that sperm–ZP interaction may be facilitated, at least in part, by the core polypeptide backbone of ZP3 (Florman et al. 1984; Chapman et al. 1998; Hinsch et al. 2005). Specifically, the polypeptide backbone has been implicated in the induction of acrosomal exocytosis (Chapman et al. 1998; Hinsch et al. 2005). This notion is consistent with the demonstration that although sperm are able to bind to the glycocalyx of rabbit erythrocytes in a manner that appears analogous to that of ZP binding, this interaction fails to elicit the signaling cascades required to induce an acrosome reaction (Clark et al. 1996; Clark and Dell 2006). Notably, phenotypic analysis of a number of transgenic mouse models have also raised the prospect that the three-dimensional structure of the zona matrix, rather than a single protein (or carbohydrate), may be central to mediation of sperm binding (Dean 2004, 2005; Hoodbhoy and Dean 2004).

2.1.4 Models of ZP3 Independent Sperm–ZP Interaction

The debate regarding the nature of the ZP3 ligand(s) responsible for initiating sperm–egg interaction has been overshadowed by recent evidence that sperm may be able to resolve gamete recognition into at least two distinct binding events. In this context, the work of Shur and colleagues has raised the interesting prospect that prior to engaging in interaction with ZP3 ligands, sperm are able to be tethered to the oocyte via adhesion to an oviduct-derived glycoprotein (oviduct-specific glycoprotein, OGP) (Rodeheffer and Shur 2004; Lyng and Shur 2009). OGP is a high molecular weight glycoprotein synthesized and secreted by oviductal cells within the fimbriae and infundibulum and apparently coats the periphery of the ZP in addition to permeating the perivitelline space of ovulated mouse oocytes (Rodeheffer and Shur 2004; Lyng and Shur 2009). Support for this ZP3 independent model has been advanced by the demonstration that both immunoprecipitated and natively purified OGP are able to competitively inhibit sperm–egg binding (Lyng and Shur 2009). The sperm-binding activity of OGP appears to be carbohydrate-dependent since denatured OGP retains the ability to inhibit binding, and interestingly is restricted to a relatively minor peanut agglutinin (PNA)-binding glycoform (Lyng and Shur 2009). While such findings may initially appear to be at odds with data from competitive inhibition assays indicating that mouse sperm–oocyte interaction is potently inhibited by preincubation of the sperm with either solubilized ZP or purified mouse ZP3 (Bleil and Wassarman 1980a), it must be remembered that these latter experiments were conducted *in vitro* and therefore may not be entirely physiologically relevant. It is also noteworthy, however, that ectopic ovarian pregnancies, although rare, have been recorded in humans (Cabero et al. 1989). These pregnancies take place without the oocyte ever reaching the oviduct and therefore would be unlikely to have been exposed to OGP.

2.1.5 The Role of ZP2 in Sperm–ZP Interactions

The role of ZP2 during gamete interaction has traditionally been viewed as that of a secondary ligand that possesses the ability to bind to the inner acrosomal membrane of acrosome-reacted sperm, thus ensuring close contact between the penetrating spermatozoon and the zona matrix (Bleil et al. 1988). This sperm-binding affinity of ZP2 is abrogated by the proteolytic modification of the protein that accompanies cortical granule exocytosis at the moment of fertilization (Moller and Wassarman 1989). The modification of ZP2 facilitates an increase in the interaction between the ZP filaments, in turn promoting the hardening of the ZP (Moller and Wassarman 1989) and thus producing one of two blocks to polyspermy. An interesting caveat to this model has recently been advanced by the work of Dean and colleagues using transgenic mice expressing a chimeric zona pellucida, containing human ZP2 (Dean 2004). The oocytes of these mice bind mouse but not human spermatozoa. Surprisingly, however, this sperm-binding activity persists even after fertilization of the oocytes. This phenomenon is not completely understood, since the ZP2

proteolytic cleavage domain is conserved between species and the human ZP2 would therefore have been expected to be digested following cortical granule exocytosis (Dean 2004, 2005). Interestingly, similar results were also observed in mice expressing human ZP3. One possible explanation for these anomalous results is that sperm binding is modulated by the overall supramolecular structure of the zona pellucida rather than relying on individual proteins and/or oligosaccharides (Dean 2004).

2.2 Sperm Receptor Molecules Involved in Zona Pellucida Interaction

In accordance with the complexity of the various models for sperm–zona interaction, it has proven difficult to identify definitively the corresponding sperm surface molecules that mediate primary recognition and adhesion to the ZP. A multiplicity of putative ZP receptors have been postulated on the basis of a range of experimental techniques including analysis of mutations influencing fertility, development of inhibitory monoclonal antibodies, analysis of sperm autoantigens, ZP affinity columns, photoaffinity crosslinking, and binding of radiolabeled ZP to sperm lysates (reviewed by McLeskey et al. 1998; Table 1). Consistent with the model of primary sperm–egg interaction being initiated by defined carbohydrate structures on ZP3 (Sect. 2.1.1), a number of these putative receptors possess lectin-like affinity for specific sugar residues (Table 1). However, notwithstanding compelling *in vitro* data implicating these molecules in zona adhesion, no single candidate has been identified that is uniquely responsible for directing the interaction between sperm and the ZP. Such findings fuel speculation that this fundamental cellular interaction may require the coordinated action of several sperm proteins. Indeed, emerging evidence supports the concept that sperm–zona interaction is mediated by a multimeric complex incorporating several discrete molecular entities, each of which may have a specific role at different stages of the recognition process (see Sect. 2.2.2). Furthermore, in recognition of the fact that gamete interaction is predicated on spermatozoa acquiring a state of functional maturation during their post-testicular development, it has been suggested that the assembly of this complex may be causally linked to membrane remodeling events associated with epididymal maturation and/or capacitation (Sect. 2.2.1).

2.2.1 Acquisition of the Ability to Engage in Sperm–ZP Interaction

Mammalian spermatozoa are produced by spermatogenesis, a prolonged, inordinately complex process that culminates in the generation of a morphologically mature, yet functionally incompetent sperm cell (reviewed by Eddy and O'Brien 1994). During this process, spermatids undergo a dramatic metamorphosis from a rounded shape into an elongated cell consisting of a number of highly specialized

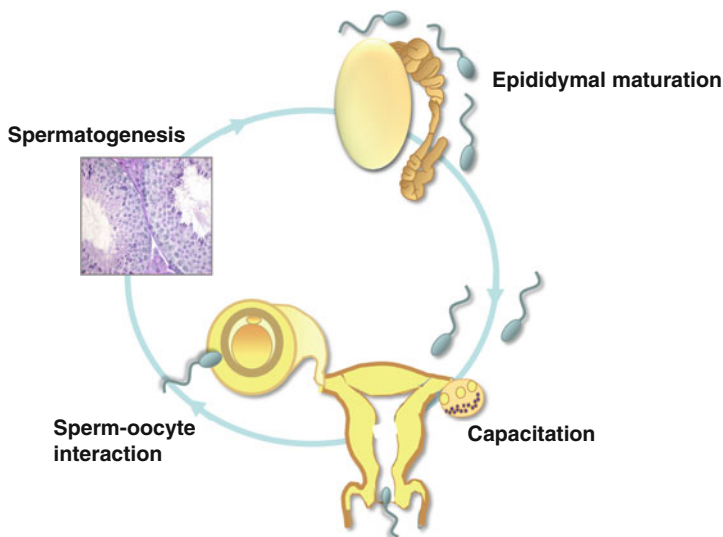


Fig. 2 Phases of sperm maturation required for successful sperm–oocyte interaction. Following their production in the testes (spermatogenesis), mammalian spermatozoa enter the male reproductive tract (epididymis) as functionally incompetent cells. Exposure to the intraluminal milieu of the epididymis results in acquisition of the potential for forward progressive motility and the ability to engage in interaction with the ZP. However, these functional attributes are only expressed after a final phase of maturation (capacitation) as the spermatozoa ascend the female reproductive tract

regions: a head comprising the acrosomal vesicle, nucleus, cytoskeletal structures, and cytoplasm; a midpiece which houses the mitochondria; and a flagellum that is used for locomotion. The final phase of cytodifferentiation, spermiogenesis, is also characterized by the repackaging of the chromosomes in preparation for their delivery to the oocyte (Eddy and O'Brien 1994). As a consequence of these events, it is widely held that spermatozoa leave the testes in a transcriptionally silent state and similarly lack the capacity for *de novo* protein synthesis (Engel et al. 1973). The post-testicular functional transformation of these cells that ensues is therefore reliant upon protein changes (loss, acquisition and post translational modification) driven by exposure to the external environment, as these cells move through the male and female reproductive tracts (Fig. 2).

Epididymal Maturation

Notwithstanding the high degree of morphological specialization that is achieved during spermatogenesis, spermatozoa enter the epididymis without the capacity to exhibit forward progressive motility nor to recognize and engage in interaction with the ZP (reviewed by Yanagimachi 1994). Spermatozoa acquire the potential to express these functional attributes during their transit of the male reproductive tract,

particularly the epididymis (reviewed by Cooper 1986). Elegant ligation and epididymostomy studies have provided compelling evidence that the accompanying changes are not intrinsic to spermatozoa, but rather appear to be driven by dynamic changes in the ambient intraluminal milieu as they pass along the length of the epididymal tubule (Cooper 1986). Indeed, the exposure of spermatozoa to the microenvironment created by the combined secretory and resorptive functions of the epididymal epithelial cells has been variously correlated with the addition, repositioning, removal, and/or modification of specific proteins and lipids within the sperm membrane (Jones 1998, 1999; Jervis and Robaire 2001; Chaurand et al. 2003; Johnston et al. 2005; Turner et al. 2006).

The ability of epididymal spermatozoa to bind to the ZP is first observed in the proximal corpus epididymis and achieves maximal levels in the caudal region in virtually all species studied to date (Cooper 1986). Interestingly, the acquisition of zona binding competence coincides with the attainment of the potential for movement (Aitken et al. 2007). However, it is considered unlikely that these two events are causally related since, unlike motility, sperm–zona interaction is dependent on the ability of the spermatozoa to undergo capacitation, with noncapacitated cells proving largely refractory to zona adhesion (Asquith et al. 2004). Additionally, zona binding ability is retained in immobilized caudal epididymal spermatozoa (Saling 1982). The acquisition of zona binding also appears temporally associated with the exposure of spermatozoa to two distinct subsets of macromolecular structures in the epididymal lumen: the first being amorphous, chaperone laden “dense bodies” (Asquith et al. 2005) and the second being membrane-bound prostasome-like particles known as epididymosomes (Saez et al. 2003). It has been hypothesized that together, these epididymal granules facilitate the bulk transfer of proteins to the sperm surface during their transit of the organ. This idea is consistent with the demonstration that biotinylated proteins are able to be transferred between epididymosomes and the acrosomal cap and midpiece of spermatozoa (Saez et al. 2003). However at present, neither the molecular mechanisms that underpin protein transfer nor the identity of the transferred protein(s) has been fully elucidated. Similarly, the causative nature of this relationship remains the subject of ongoing investigation.

Sperm Capacitation

Following their passage through the epididymis spermatozoa must complete an additional phase of maturation, termed capacitation, before realizing their full potential for fertilization. Capacitation occurs *in vivo* as spermatozoa ascend the female reproductive tract and encompasses a series of elaborate cellular modifications. Indeed, in the 60 years that have elapsed since capacitation was first described (Austin 1951; Chang 1951), a number of changes have been correlated with this process, including extensive remodeling of the sperm plasma membrane and the posttranslational modification of intrinsic sperm proteins (Visconti et al. 1995a, b; Gadella and Van Gestel 2004; Boerke et al. 2008; Gadella 2008; Gadella et al. 2008).

Among the posttranslational modifications that have been documented to date, a global upregulation of phosphotyrosine expression has emerged as a critical factor in regulating the ability of spermatozoa to hyperactivate, bind to the zona pellucida, undergo an acrosome reaction, and ultimately fertilize the oocyte (Visconti et al. 1995a, b, 2002; Luconi et al. 1998; Urner et al. 2001; Sakkas et al. 2003; Asquith et al. 2004; O'Flaherty et al. 2005; Baker et al. 2006; Mitchell et al. 2007).

The induction of tyrosine phosphorylation appears to be modulated predominantly by a unique soluble adenylyl cyclase/cAMP/PKA axis (Visconti et al. 1995a, b; Aitken et al. 1998). However, in addition to PKA-dependent phosphorylation of targets which for the most part appear to reside within the sperm flagellum (Visconti et al. 1997; O'Flaherty et al. 2005; Baker et al. 2006; Mitchell et al. 2007), an alternative subset of tyrosine phosphorylated proteins have been detected on the surface of live, capacitated mouse spermatozoa (Asquith et al. 2004; Piehler et al. 2006). Furthermore, the expression of these proteins appears confined to the plasma membrane overlying the acrosomal domain of the sperm head – an ideal position from which to orchestrate the membrane remodeling events associated with sperm–ZP recognition. In contrast to the aforementioned flagellar proteins, we have recently secured evidence that the phosphorylation of these sperm surface proteins is largely insensitive to inhibition with specific antagonists of the canonical PKA pathway (Nixon et al. 2010). Rather our findings suggest that an alternative signaling pathway involving the classical MAP kinases may underpin this capacitation-associated surface exposure of phosphotyrosine residues in mouse spermatozoa (Nixon et al. 2010). These findings take on added significance in light of the demonstration that the inhibition of the MAP kinase pathway, and hence sperm surface phosphotyrosine expression, induces a concomitant reduction in sperm–zona pellucida interaction (Nixon et al. 2010).

Although surface phosphotyrosine expression does not appear to be a universal correlate of capacitation in all species (Liu et al. 2006), it is not unique to mouse spermatozoa. For instance, recent quantitative studies of surface phosphotyrosine expression in boar spermatozoa have revealed a significant increase in phosphotyrosine-associated fluorescence following capacitation (Piehler et al. 2006). This increase coincides with the exposure of several tyrosine phosphorylated proteins on the outer leaflet of the boar sperm plasma membrane, at least two of which possess high affinity for the ZP (Flesch et al. 1999, 2001). In contrast, plasma membrane proteins isolated from freshly ejaculated boar spermatozoa did not exhibit any ZP binding proteins, likely because these proteins were not tyrosine phosphorylated (Flesch et al. 1999, 2001). Unfortunately however, the identity of these proteins remains to be elucidated.

Interestingly, our own analysis of the repertoire of phosphoproteins that are uniquely expressed on the surface of capacitated mouse spermatozoa, identified a subset of molecular chaperone proteins including heat shock 60 kDa protein 1 (HSPD1) and heat shock protein 90, beta 1 (HSP90B1) (Ecroyd et al. 2003; Asquith et al. 2004). Both of these proteins have in turn been localized to dense bodies within the proximal corpus epididymis (see Sect. 2.2.1) and to the sperm surface overlying the anterior acrosome, the precise location where sperm–zona interaction is initiated (Asquith et al. 2004, 2005). Although such findings invite speculation

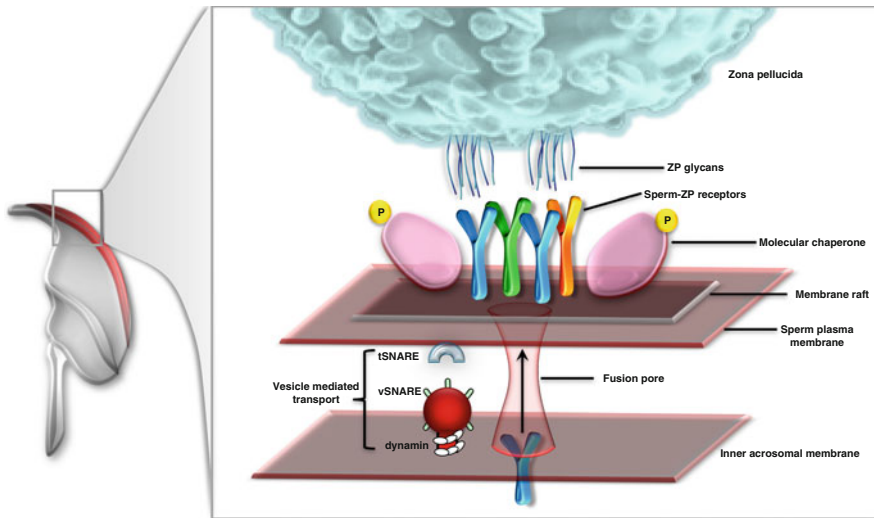


Fig. 3 *Model for mouse sperm–zona pellucida interaction.* We propose that sperm membrane rafts serve as a platform for the recruitment of key zona adhesion molecules and promote their delivery to the sperm head. This process coincides with the capacitation-associated phosphorylation of a subset of molecular chaperones and their exposure on the sperm surface. The chaperones subsequently provide the molecular machinery to assemble a functional receptor complex, rendering the sperm competent to bind to the glycans that furnish the zona pellucida. Based on emerging evidence, we also propose that capacitation-associated sperm surface remodeling may be underpinned by the translocation of a subset of ZP receptors from the acrosomal vesicle to the sperm membrane. At least two mechanisms, i.e., vesicle-mediated transport and the formation of small fusion pores, have been postulated to account for the incremental exposure of these proteins

that these chaperones may directly mediate sperm–egg interaction, our cumulative evidence argues against such a conclusion (Walsh et al. 2008). Rather, it is suggested that these proteins are responsible for chaperoning key recognition molecules to the site of sperm–oocyte interaction and/or orchestrating their assembly into a multimeric zona–receptor complex on the sperm surface (Nixon et al. 2005) (see Sect. 2.3.1; Fig. 3). In agreement with this model, we have recently employed the novel technique of blue native polyacrylamide gel electrophoresis (BN-PAGE) to provide the first direct evidence for the expression of chaperone laden complexes on the surface of capacitated mouse spermatozoa. Interestingly, a subset of these complexes also harbor putative ZP receptor proteins and possess strong affinity for solubilized zonae (Dun et al., unpublished).

2.2.2 ZP Receptor Candidates

Considerable research has been devoted to investigating the identity of the individual proteins in spermatozoa that facilitate the binding of this specialized cell to the ZP (reviewed by Nixon et al. 2007). On the basis of varying degrees of

circumstantial and direct evidence in excess of ten different candidate proteins have been proposed to participate in different aspects of this interaction in the mouse model alone (Table 1). Consistent with the notion that primary sperm–zona pellucida binding is a carbohydrate-mediated event, a number of these candidate sperm proteins are either glycoenzymes or possess the requisite lectin-like affinity for ZP3 sugars.

Perhaps the most widely studied of the putative ZP3 receptor candidates is mouse β -1,4-galactosyltransferase (GalTase). This enzyme normally resides within the Golgi apparatus, where it functions in the biosynthesis of complex glycoconjugates on secretory and membrane-bound glycoproteins (Nixon et al. 2001). However a novel, functionally distinct, isoform of GalTase has been shown to be expressed during spermatogenesis and localized to the dorsal, anterior aspect of the membrane overlying the intact acrosome (Shur and Neely 1988). From this position, GalTase is thought to function as a gamete receptor by binding to complementary terminal *N*-acetylglucosamine (GlcNAc) residues that furnish the Sperm Combining Site of the ZP3 protein (Shur and Hall 1982; Shur and Neely 1988; Shur 1989; Miller et al. 1992). Furthermore, aggregation of GalTase by ZP3 oligosaccharides activates a heterotrimeric G-protein coupled signaling cascade that culminates in the induction of the acrosome reaction (Macek et al. 1991; Miller et al. 1993). Accordingly, overexpression of GalTase on the sperm surface leads to increased ZP3 binding, accelerated G-protein activation, and precocious acrosome reactions (Youakim et al. 1994).

The expression of GalTase isoforms in the anterior portion of the sperm head of a variety of mammalian species raises the possibility that the zona receptor activity of the protein may be widely distributed (Humphreys-Beher and Blackwell 1989; Sullivan et al. 1989; Larson and Miller 1997). Surprisingly, however, targeted mutations of mouse GalTase do not induce the anticipated infertility phenotype (Lu and Shur 1997). Rather, spermatozoa from GalTase null males retain their fertility despite a marked reduction in their ZP3 binding affinity and an inability to undergo a ZP3 induced acrosome reaction (Lu and Shur 1997). Thus, although the ZP3–GalTase receptor–ligand complex may confer a physiological advantage on fertilizing spermatozoa, its expression is dispensable for fertilization. Similarly, definitive studies examining the effects of null mutations on additional sperm surface components that have been implicated in ZP adhesion have shown that the majority are also superfluous (see Table 1). Collectively these findings raise the intriguing possibility, discussed below, that the zona receptor is in fact a multimeric complex incorporating several discrete molecular entities.

Molecular Basis for Multiple Sperm–ZP Receptor Candidates

Despite the significant advances in our understanding of the initial interaction between sperm and the oocyte, it is clear that this fundamental recognition event remains largely enigmatic. The large number of sperm molecules that possess affinity for the ZP (Table 1) challenges the concept of a simple lock and key

mechanism to account for gamete interaction. Indeed, despite the wealth of in vitro data implicating various ZP receptor candidates, the prevailing evidence now indicates that none are uniquely responsible for directing the interaction between sperm and the ZP (Nixon et al. 2007). The fact that spermatozoa are adorned with a multiplicity of ZP receptor candidates could afford the cells with a level of functional redundancy commensurate with the overall importance of this fundamental cellular interaction. However, it is also possible that the individual receptors function in a coordinated fashion, each with unique role(s) during the multifaceted ZP recognition process. The latter model is consistent with biochemical and biophysical studies of sperm–ZP binding that indicate it comprises both low and high affinity interactions (Thaler and Cardullo 1996, 2002). Indeed, prior to penetration of the ZP, spermatozoa first adhere loosely to the zona matrix in a manner that is easily disrupted by repetitive pipetting or density gradient centrifugation (Bleil and Wassarman 1980a). The promiscuous nature of this initial binding event contrasts with the high affinity, comparatively species-specific, interaction that follows. The former adhesion event is therefore likely to employ sperm surface molecules that are conserved across species, while those involved in the latter binding event are, instead, expected to be species- and/or order-specific (Tanphaichitr et al. 2007). In addition to those sperm proteins required for zona adhesion, alternative candidates may be engaged in the activation of ZP-induced sperm signaling events that culminate in the acrosome reaction. If this model holds true, it inevitably raises questions regarding how the presentation of such a large number of putative ZP receptor and signal transduction candidates is coordinated. As discussed below, recent analyses have led to the proposal of at least two complementary mechanisms involving molecular chaperones and membrane rafts, the relative contribution of which may vary depending on the species.

2.3 Toward an Integrated Model of Sperm–Zona Interaction

2.3.1 The Role of Molecular Chaperones in Sperm–Zona Pellucida Interaction

The term molecular chaperone denotes a large family of highly conserved proteins that form a ubiquitous defense system within cells. However, in addition to their archetypal role of protecting cells from the adverse effects of stress, it has become increasingly apparent that chaperones play additional roles in diverse cellular phenomena under normal physiological conditions. Of particular note is the recognized ability of molecular chaperones to direct the assembly of oligomeric protein complexes and mediate their transport across the plasma membrane (Voos and Rottgers 2002). In addition, emerging evidence indicates that certain members of the chaperone family exert a necessary, but still poorly understood, role in the recruitment and clustering of specific receptors on the cell surface and in signal transduction (Triantafilou et al. 2001; Triantafilou and Triantafilou 2003, 2004).

Consistent with such roles, chaperones have been identified within a number of divergent subcellular compartments including the plasma membrane of a wide variety of cell types (Soltys and Gupta 1996, 1997, 1999; Shin et al. 2003). Interestingly, the chaperoning activity of the plasma membrane resident chaperones appears to be regulated by their phosphorylation status (Khan et al. 1998). The significance of this finding is underscored by the demonstration that at least two key chaperone proteins are phosphorylated during the capacitation of mouse spermatozoa (Asquith et al. 2004) (see Sect. 2.2.1).

Although several molecular chaperones, including calmegin, calnexin, and members of the HSP60, 70, and 90 families, have been identified in spermatogenic cells (Tanaka et al. 1997; Zhu et al. 1997; Eddy 1998; Ohsako et al. 1998; Ogi et al. 1999; Yoshinaga et al. 1999), the functional significance of many of these proteins remains unclear. However, at least one member of this family, calmegin, has been implicated in sperm–ZP interaction (Ikawa et al. 1997, 2001; Yamagata et al. 2002). Despite the fact that calmegin is not expressed in mature spermatozoa, it has been identified as a critical determinate in the functioning of these cells on the basis of its role in ensuring the correct folding of endoplasmic reticulum glycoproteins destined for the acrosomal matrix and the plasma membrane. Targeted disruption of the calmegin gene compromises male fertility due to impaired sperm transport in the female reproductive tract in vivo (Ikawa et al. 2001) and the loss of sperm–zona binding ability (Ikawa et al. 1997). An absence of signaling proteins or antigenic determinants from the surface of sperm has been proposed as the mechanism to explain these defects in sperm function. Interestingly, in this regard, sperm from calmegin^{-/-} mice also lack fertilin beta (ADAM2), a protein implicated in sperm–egg plasma membrane binding and fusion (Ikawa et al. 2001). Thus, the chaperone function of calmegin may regulate the correct processing of a variety of sperm molecules. Collectively, such observations invite speculation that chaperones direct the assembly of key recognition molecules on the sperm surface.

Support for this hypothesis rests with the demonstration that mouse spermatozoa express a subset of molecular chaperones (including: HSPE1, HSPD1, HSP90, and HSP90B1) within the periacrosomal region of their head (Ecroyd et al. 2003; Asquith et al. 2004; Walsh et al. 2008). Interestingly, the surface expression of these proteins increases dramatically in populations of sperm in which capacitation has been actively driven. Nonetheless, a direct role for the chaperones in sperm–oocyte interaction has been discounted on the basis that incubation of sperm with anti-chaperone antibodies does not significantly compromise their ability to bind to the ZP (Walsh et al. 2008). Rather it appears that chaperones play an indirect role possibly in the assembly of multiple zona adhesion molecules into a functional receptor complex (Fig. 3). An alternative possibility is that molecular chaperone proteins participate in the active translocation of sperm proteins to their site of action.

Since spermatozoa lack the molecular machinery for protein synthesis, these proteins must be either unmasked or held cryptic within the cell prior to their surface presentation. A growing body of evidence favors the latter interpretation, indeed many of the putative ZP receptors are proteins one would normally

associate with the sperm acrosome (Tulsiani and Abou-Haila 2001, 2004). The zona pellucida 3 receptor (ZP3R; formerly sperm protein 56 or SP56) provides an interesting example of one such protein. ZP3R was originally identified on the basis of elegant photoaffinity crosslinking studies as a primary receptor for ZP3 (Bleil and Wassarman 1990; Cheng et al. 1994). This role was subsequently discounted on the basis of immunoelectron microscopy evidence that revealed the protein was enclosed within the acrosomal matrix (Foster et al. 1997; Kim et al. 2001a). Since such a location is incompatible with the mediation of ZP3 binding in acrosome intact spermatozoa, it was postulated that the ZP3R was likely to participate in secondary sperm–ZP interactions. Resolution of this apparent discrepancy has recently been afforded by the demonstration that ZP3R, in addition to other acrosomal matrix proteins, are progressively released to the sperm surface during capacitation through the formation of small fusion pores (Fig. 3) (Kim et al. 2001b; Kim and Gerton 2003; Buffone et al. 2008b). This evidence not only challenges the widely held view of acrosomal exocytosis as an all or none reaction but also raises the intriguing possibility that the acrosome may fulfill a secondary role as a reservoir for key ZP recognition molecules (Buffone et al. 2008a). This interpretation may explain why uncapacitated mammalian sperm are unable to engage in high affinity interaction with the ZP. However, while it is tempting to speculate that chaperones mediate the relocalization of these proteins and hence prime the sperm surface for ZP adhesion, direct evidence in support of this model has yet to be furnished.

Among the main challenges that remain in establishing the definitive role of molecular chaperones in mature spermatozoa is the characterization of the client proteins with which they associate in both noncapacitated and capacitated spermatozoa (Nixon et al. 2007). Unfortunately, the use of conventional techniques such as affinity purification and immunoprecipitation has proven largely unsuccessful in this regard (Walsh et al. 2008). This lack of success may reflect the fact that chaperones generally form only weak, transient interactions with their client proteins. Among the alternative strategies that could prove informative in this regard are the isolation and detailed proteomic characterization of the repertoire of membrane-associated proteins that populate the region of the sperm head that interacts with the oocyte. Such an approach has been published by Myles and colleagues (Stein et al. 2006). Following vectorial labeling of the mouse sperm surface, the authors conducted a comparative analysis of the profile of surface exposed proteins with that of proteins recovered in hybrid membrane vesicles released from the anterior sperm head following the acrosome reaction (Stein et al. 2006). This approach has helped define the basic proteomic inventory of the anterior sperm head, the significance of which is highlighted by the fact that among the 85 proteins identified were at least three molecular chaperones (including HSP90B1) in addition to nine proteins that have been implicated in fertilization *in vivo* on the basis of gene knockout studies (Stein et al. 2006). One limitation of this approach, however, was that it did not address the important question of the temporal and spatial organization of membrane-associated proteins in relation to the dynamic cellular changes that accompany capacitation.

A complementary strategy has recently been published by Gadella and colleagues (van Gestel et al. 2007). In this study, the authors isolated the apical plasma membrane from porcine sperm by nitrogen cavitation, achieving an approximate 20-fold enrichment in plasma membrane markers compared with that of contaminating membrane markers. These membrane preparations were then coincubated with isolated zona ghosts and sperm-ZP binding proteins were identified by tandem mass spectrometry (van Gestel et al. 2007). This study confirmed the involvement of multiple sperm proteins in ZP binding, with 24 sperm proteins reproducibly remaining associated with zona ghosts under conditions of low stringency. As anticipated, a subset of these proteins was identified as previously characterized ZP-binding receptors including: spermadhesin (AQN-3), P47 (SED1), and fertilin beta (ADAM2). Remarkably, the majority of the zona ghost-binding proteins were also detected in lipid ordered membrane microdomains (membrane rafts) that are assembled in the apical ridge area of the sperm head plasma membrane during *in vitro* capacitation (Boerke et al. 2008). On the basis of such evidence, it has been postulated that the study of membrane rafts may provide novel insights into the molecular mechanisms that underpin sperm-ZP interaction (Tanphaichitr et al. 2007; Gadella 2008; Gadella et al. 2008; Nixon and Aitken 2009).

2.3.2 The Role of Membrane Rafts in Sperm-Zona Pellucida Interaction

Membrane rafts (formerly lipid rafts) are generally defined as small, heterogeneous domains that serve to compartmentalize cellular processes (Pike 2006). The unique, ordered properties of these domains reflect the stabilizing influence of hydrogen bonds and hydrophobic interactions between their resident saturated fatty acids and the rigid structure of intercalated cholesterol. These properties also result in the resistance of lipid rafts to solubilization by a number of nonionic detergents (Schuck et al. 2003) and hence they are often referred to as detergent resistant membranes (DRMs). Despite their stability, rafts remain highly dynamic and have been observed to display considerable lateral movement in various cell types in response to appropriate physiological stimuli or cellular activation events (Simons and Vaz 2004). The significance of these structures is highlighted by the myriad of cell adhesion, signaling and trafficking molecules that have been found to preferentially associate with isolated membrane rafts (Foster et al. 2003). Indeed membrane rafts are now considered as platforms for mediating membrane trafficking, cellular signal transduction, and cellular adhesion events as diverse as viral entry and fertilization (Nixon and Aitken 2009).

It has been demonstrated that liquid-ordered domains analogous to the membrane rafts observed in somatic cells are present in the spermatozoa of all mammalian species studied to date, albeit at a larger scale (Cross 2004; Shadan et al. 2004; Sleight et al. 2005; Bou Khalil et al. 2006; Selvaraj et al. 2006, 2009; Weerachatanukul et al. 2007; Boerke et al. 2008; Asano et al. 2009; Nixon et al. 2009). Indeed, the

size and stability of sperm membrane rafts appear quite excessive, raising the possibility that they may represent “super-rafts” consisting of stably segregated smaller subdomains (Selvaraj et al. 2006, 2009). This is consistent with a recent demonstration that a number of subtypes of membrane raft domains are likely to exist in these cells (Asano et al. 2009). With the recognition that mammalian spermatozoa possess membrane rafts, two lines of enquiry have predominated. First, whether the physical and biochemical properties of the membrane rafts are influenced by the capacitation status of spermatozoa, and second, whether the rafts modulate important aspects of sperm function (Tanphaichitr et al. 2007). Paradoxically, two conflicting views have emerged from the former studies, with evidence suggesting that these domains may either be compromised by the capacitation-associated loss of cholesterol (Sleight et al. 2005), or alternatively may cluster within the sperm head and coalesce to form larger ordered membrane microdomains during capacitation (Shadan et al. 2004; Bou Khalil et al. 2006; Boerke et al. 2008; Nixon et al. 2009). It is still unclear what functions might underlie such distinct membrane remodeling; however, the focal enrichment of membrane rafts within the sperm head encourages speculation that they may serve as platforms for modulating oocyte interaction (Fig. 3) (Tanphaichitr et al. 2007). This hypothesis is commensurate with the demonstration that DRMs isolated from both boar and mouse spermatozoa possess the ability to bind with high affinity and specificity to the zona pellucidae of homologous oocytes (Bou Khalil et al. 2006; Boerke et al. 2008; Nixon et al. 2009).

Consistent with these findings, comprehensive proteomic profiling of isolated sperm DRMs has confirmed the anticipated presence of the majority of molecules that have been implicated in sperm–zona pellucida binding (Table 1) in addition to many of those involved in downstream interaction with the oolemma (Sleight et al. 2005; Nixon et al. 2009). Although caution is required in equating DRM association with a protein’s residence in membrane raft domains *in situ* (Foster et al. 2003; Munro 2003), such findings suggest that sperm membrane rafts serve as constitutive platforms for the spatial constraint of key recognition molecules and that the remodeling events associated with capacitation lead to their assembly and presentation on the outer leaflet of the sperm plasma membrane (Nixon et al. 2005, 2007).

Such a conclusion is supported by the demonstration that the proteomic composition of membrane rafts undergoes substantial changes in response to the induction of capacitation (Thaler et al. 2006). Among the various models that could account for such changes, Tulsiani and colleagues (Abou-Haila and Tulsiani 2003) have postulated that capacitating spermatozoa undergo a progressive priming that results in the exposure of intraacrosomal enzymes (see Sect. 2.3.1). Their model is based on the precept that as capacitation proceeds, the outer acrosomal membrane evaginates, forming a vesicle that enlarges and becomes tethered to the plasma membrane through complementary vesicle-associated (v-) SNARE and target membrane (t-) SNARE proteins residing within the two membranes (Fig. 3). Although the movement of vesicles between the sperm acrosome and plasma membrane has not previously been documented during capacitation, many of the components of the molecular machinery necessary for coordinating the assembly and trafficking of

exocytotic vesicles are present in spermatozoa. For instance, dynamin, an enzyme that forms restriction collars around budding vesicles and promotes their release, has recently been identified within the acrosome of mouse spermatozoa (Zhao et al. 2007). Furthermore, the complementary SNARE proteins, VAMP, SNAP, and syntaxin have been localized to the outer acrosomal membrane and apical sperm head plasma membrane, respectively, of mouse spermatozoa (Brahmaraju et al. 2004; Tsai et al. 2007) in addition to that of other species (Schulz et al. 1998; Tomes et al. 2002; De Blas et al. 2005). Notably these SNARE proteins share the lateral redistribution properties of both ZP-binding proteins and raft marker proteins, each of which are able to be recovered within DRMs prepared from capacitated spermatozoa (Boerke et al. 2008). Collectively, these data invite speculation that key components of a zona adhesion complex are conveyed from the acrosomal vesicle into membrane rafts during capacitation. This notion is supported by the demonstration that antibodies to VAMP and SNAP inhibited mouse sperm–zona pellucida interaction (Brahmaraju et al. 2004).

It is also noteworthy that sperm membrane rafts are laden with a subset of molecular chaperone proteins (Nixon et al. 2009), at least two of which, HSP90B1 and HSPD1, have previously been implicated in remodeling the sperm surface and enhancing oocyte interaction (Asquith et al. 2004, 2005; Sect. 2.2.1). The identification of a number of constitutively expressed molecular chaperones (HSP90B1, HSPA8, HSPD1, and DNAJB1) as integral components of membrane rafts in other cell types (Broquet et al. 2003; Chen et al. 2005) suggests that these proteins may fulfill an important general mechanism operating at the level of the plasma membrane through which cellular signaling/adhesion complexes are sorted and assembled. In this regard, previous studies have demonstrated that chaperones play important roles in maintaining the stability of lipid raft-associated signal transduction complexes (Chen et al. 2005). Conversely, it has also been demonstrated that lipid rafts regulate the functions of resident chaperones through the spatial constraint of their substrates (Elhyany et al. 2004). Taken together these results suggest that sperm membrane rafts provide a favorable environment for chaperones to mediate the conformational conversion and assembly of functional zona receptor complexes. Furthermore, the aggregation of such microdomains during capacitation may facilitate the recruitment of these complexes to the site of engagement with the ZP.

3 Potential for Contraceptive Intervention

In addition to fundamental benefits in terms of understanding causes of male infertility, the molecular dissection of sperm–ZP interaction also promises to inform the development of novel approaches for contraceptive intervention. Indeed, it has long been held that the identification of sperm proteins involved in ZP recognition and binding events could provide a range of candidates that, by virtue of their specificity, location, and susceptibility to suppression, would exhibit

potential as contraceptive targets with equal effectiveness for both males and females. Such contraceptives would be of considerable benefit for the control of both captive and feral animal species and thus contribute to ameliorating global problems associated with a lack of habitat, overcrowding, and disease (Hardy and Braid 2007; Kirkpatrick 2007; Fayer-Hosken 2008). The realization of such technology may also contribute to the development of novel, safe, effective measures to fill the void in the current contraceptive armory for our own species, the population of which continues to grow at an alarming rate (McLaughlin and Aitken *in press*). Contraceptive vaccines, for example, have the potential to provide safe, effective, prolonged, reversible protection against pregnancy in a form that can be easily administered in the Third World. However, in order to meet the above criteria, the target antigen must be an essential component of fertility and must be inhibitable.

3.1 Target Antigens of the Zona Pellucida

The zona pellucida glycoproteins are among the most widely investigated candidate targets for immunocontraceptive vaccines. The ZP proteins afford the advantage of being a female organ-specific antigen and an immune response elicited against these proteins could in principle block sperm–ZP interaction. In practice, however, the reduction in fertility achieved following immunization with homologous or heterologous zona proteins appears to be primarily attributed to either the loss of endogenous antigen and/or the induction of ovarian-specific autoimmune disease (Paterson et al. 2000). This is highlighted by fertility trials involving whole native porcine zona pellucida (pZP), a popular heterologous antigen in current use for feral and exotic animal fertility control (Hardy and Braid 2007). Such studies have demonstrated that pZP is a potent heteroimmunogen in most species and is efficacious in the control of fertility in horses, white tailed deer, bonnet monkeys, wallaby, bears, and elephants (Bagavant et al. 1994; Fayer-Hosken et al. 1999; Miller et al. 2000; Kitchener et al. 2002, 2009; Turner et al. 2002; Delsink et al. 2007; Lane et al. 2007; Locke et al. 2007). However, the use of this material as an immunogen is problematical as it has been shown to induce ovarian pathology, the loss of hormone-dependent behavior, and permanent sterility (Dietl et al. 1982; Drell et al. 1984; Bhatnagar et al. 1992). The isolation of a consistent native pZP product, free of viral contamination, for immunization purposes has also proven technically challenging (Kaul et al. 1996). While the latter problem may be alleviated by the production of glycosylated porcine ZP recombinant protein in a defined mammalian cell line, the permanent ovarian pathology that accompanies the active immunity against ZP antigens represents a significant barrier to their clinical use (McLaughlin and Aitken *in press*). Until researchers separate the immunocontraceptive effect from the unwanted pathology induced by immunodominant epitopes, ZP proteins will remain unlikely target antigens for a human immunocontraceptive vaccine. Furthermore, the fact that ZP immunogens lack

species-specificity imposes restraints on their mode of delivery and hence overall applicability in free-ranging wildlife species. Other antigenic targets are clearly required.

3.2 *Target Antigens of Spermatozoa*

The demonstration that spermatozoa are highly immunogenic in both females and males presents a strong rationale for the development of contraceptive technologies centered on a defined sperm-specific antigen. This is emphasized by the fact that the presence of antisperm antibodies in the male or female partner has been identified as a causative agent in the infertility associated with a relatively large number (9–36%) of couples seeking recourse to assisted conception (Menge et al. 1982; Collins et al. 1993; Ohl and Naz 1995). Furthermore, the development of antisperm antibodies that occurs in over 70% of vasectomized men limits the potential for recovery of fertility even after successful vaso-vasostomy surgery (Hull et al. 1985).

Notwithstanding the award of a US patent for a spermatotoxic vaccine based around the injection of whole semen (Baskin 1932), this approach has limited utility. Among the obvious problems is the fact that spermatozoa express numerous antigens that are shared with somatic cells, thus raising the prospect of potentially severe immunopathological side effects. Attention has instead focused on the identification of individual sperm proteins capable of eliciting a contraceptive response. The appropriate sperm antigen should display sperm-specific expression, surface accessibility and have a pivotal role in fertilization. In principle, sperm proteins involved in ZP interaction therefore represent ideal candidates. Accordingly, a myriad of these proteins, including: SP17 (O'Rand and Widgren 1994), SPAM1 and ADAM1/2/3 (Primakoff et al. 1987, 1988; McLaughlin et al. 1997, 2001), LDHC4 (Goldberg 1973; Goldberg et al. 1981; Chen et al. 2008), SP10 (Srinivasan et al. 1995), ZPR3 (Hardy and Mobbs 1999), FA1 (Naz and Wolf 1994; Naz and Zhu 1998), SOB2 (Lefevre et al. 1997), a novel form of CD52 (Diekman et al. 1999), human sperm-associated antigen 9 (hSPAG9) (Jagadish et al. 2006), and nuclear autoantigenic sperm protein (tNASP) (Wang et al. 2009), have been investigated as the basis for a fertility-regulating vaccine. Nevertheless, while some of these antigens have shown promise in animal trials with notable inhibition of sperm–ZP interaction and concomitant subfertility, such studies have failed to deliver on the objective of identifying a single, suitable target that induces a 100% block to fertility.

Collectively, this lack of success highlights the naivety of the paradigm that sperm–ZP interaction is regulated by a single molecular entity that is constitutively expressed on the cell surface. Rather, it is likely that multiple sperm receptors are required to achieve high affinity binding to the complex multivalent polysaccharide ligands present within the ZP (see Sect. 2.2.2). The growing acceptance of this model is demonstrated by the fact that researchers have recently opted for construction of multiantigen vaccines. Examples include multiantigen recombinant

polypeptides comprising the mouse reproductive antigens SP56, ZP3, ZP2, and ZP1 administered to female mice (Hardy et al. 2008). This resulted in significantly reduced fertility without significant ovarian pathology (Hardy et al. 2008). Using six sperm-specific antigens (mFA-12,19, mFA-1117136, YLP12, P10G, A9D, and SP56) also resulted in reduced fertility in multi-peptide vaccination studies (Naz and Aleem 2007). A third vaccine formulation comprised of five recombinant human intraacrosomal sperm proteins (ESP, SLLP1, SAMP32, SP10, and SAMP14) was used to immunize female cynomolgus monkeys, all of which developed IgG and IgA serum responses to each immunogen, indicating that a multivalent contraceptive vaccine may be a viable alternative in primates (Kurth et al. 2008).

4 Summary

Despite the myriad of putative sperm–zona pellucida adhesion molecules that have been reported, no single candidate appears uniquely responsible for mediating this important interaction. Rather than this simple lock and key mechanism, the balance of evidence favors the novel hypothesis that sperm–egg interaction is mediated by the coordinated action of several sperm receptors, each of which contribute to the high affinity and specificity of the recognition process. Furthermore, it appears that these discrete receptors are either constitutively or inducibly associated with membrane rafts following the process of sperm capacitation. The fact that these specialized membrane microdomains also accommodate a family of molecular chaperones raises the intriguing possibility that spermatozoa express a multimeric zona receptor complex that is assembled into a functional unit during capacitation. The examination of this hypothesis will provide informed insights into the molecular basis of sperm–zona pellucida interaction and may pave the way for the development of novel contraceptives for feral animals and humans and the diagnosis and treatment of infertility.

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