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The molecular basis of gamete recognition in mice and humans

Matteo A. Avella*, Bo Xiong, and Jurrien Dean

Laboratory of Cellular and Developmental Biology, NIDDK, National Institutes of Health, Bethesda, MD 20892, USA

*Correspondence address. E-mail: matteo.avella@nih.gov

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ABSTRACT: Successful fertilization heralds the onset of development and requires both gamete recognition and a definitive block to polyspermy. Sperm initially bind and penetrate the extracellular zona pellucida (ZP) that surrounds ovulated eggs, but are unable to bind the zona surrounding preimplantation embryos. The ZP of humans is composed of four (ZP1–4) and that of mouse three (ZP1–3) glycoproteins. Models for gamete recognition developed in mice had proposed that sperm bind to ZP3 glycans. However, phenotypes observed in genetically engineered mice are not consistent with this widely accepted model. More recently, taking advantage of the observation that human sperm do not bind to mouse eggs, human ZP2 was defined as the zona ligand in transgenic mouse models using gain-of-function assays. The sperm-binding site is an *N*-terminal domain of ZP2 that is cleaved by ovastacin, a metalloendoprotease released from egg cortical granules following fertilization. Proteolysis of this docking site provides a definitive block to polyspermy as sperm bind to uncleaved, but not cleaved ZP2 even after fertilization and cortical granule exocytosis. While progress has been made in defining the ZP ligand, less headway has been made in identifying the cognate sperm receptor. Although a number of sperm receptor candidates have been documented to interact with specific proteins in the ZP *in vitro*, continued fertility after genetic ablation of the cognate gene indicates that none are essential for gamete recognition. These on-going investigations inform reproductive medicine and suggest new therapies to improve fertility and/or provide contraception, thus expanding reproductive choices for human couples.

Key words: gamete recognition / zona pellucida / ZP3 glycan-release models / ZP2 cleavage model / sperm surface receptor

Introduction

Human infertility, defined as the inability to conceive after 6–12 months of unprotected sexual intercourse, affects ~14% of couples (Boivin *et al*., 2007) and is recognized by the World Health Organization as a major public health issue (http://www.who.int/reproductivehealth/publications/infertility/progress63.pdf). In one-third of patients, the cause of infertility is unknown. While some struggle to have children, others seek better control over their reproductive choices to prevent unwanted pregnancies. The world population is growing at unprecedented rates and the 6.9 billion people present in 2010 are likely to increase to 9.6 billion over the next 50 years (http://esa.un.org/wpp/Excel-Data/population.htm). Initiated by seminal descriptions of human IVF (Steptoe and Edwards, 1978) and ICSI (Palermo *et al*., 1992) significant advances continue to be reported in reproductive biology and medicine. However, there remains a compelling need to better understand the molecular basis of fertilization to develop more comprehensive therapies for infertility and improved means of family planning.

At coitus, tens of millions of sperm are deposited in the lower female reproductive tract, but relatively few progress to the ampulla of the oviduct and encounter ovulated eggs. During this transit, functionally mature sperm become capacitated and gain the ability to bind to the extracellular zona pellucida (ZP), undergo acrosome exocytosis and fertilize eggs (Bailey, 2010; Buffone *et al*., 2012). The ovulated egg is encased in a cumulus oophorus composed of a hyaluronan interspersed with cumulus cells from ovarian follicles. Fertilization reflects a cascade of events that include (1) relative taxon-specific gamete recognition; (2) sperm penetration of the egg coat followed by gamete fusion and (3) a post-fertilization block to polyspermy. The ZP not only plays pivotal roles in these events, but also serves to protect the early embryo as it passes through the oviduct prior to implantation in the uterus (Yanagimachi, 1994).

Over the last several decades, investigators seeking to understand the role of the ZP in gamete recognition have proposed models based on biochemistry, cell biology and, more recently, mouse genetics. Depending on the vertebrate model, each component of the zona matrix has been proposed as a ligand for sperm binding (Bleil and Wassarman, 1980a; Tian *et al*., 1997; Ganguly *et al*., 2010; Yonezawa *et al*., 2012) which have been summarized in a number of recent reviews (Hedrick, 2008; Shur, 2008; Ikawa *et al*., 2010; Monne and Jovine, 2011; Gupta *et al*., 2012). While acknowledging that it is likely that multiple proteins play supporting roles, we focus on those that are essential for fertilization. Although continued fertility of null mutants indicates that the cognate protein is not essential for

gamete recognition, the reverse (infertility after genetic ablation) may not provide insight into gamete recognition if the absence of the protein disrupts other requirements for fertilization (e.g. sperm mobility, formation of a zona matrix and mating behaviour). We seek to review current models to discuss their strengths and weaknesses with the hope of galvanizing research to ensure a better molecular understanding of gamete recognition at the ZP.

Composition and structure of mouse and human zonae pellucidae

Four genetic loci encode mouse and human ZP genes. However, the mouse ZP (~7 μm thick) that surrounds ovulated eggs (~80 μm diameter) is composed of only three glycoproteins, ZP1 (526 aa, 120 kD), ZP2 (599 aa, 120 kD) and ZP3 (329 aa, 83 kD) (Bleil and Wassarman, 1980b; Shimizu *et al*., 1983) because *Zp4* is a pseudogene and does not express the cognate protein (Lefievre *et al*., 2004). A thicker (~16 μm) human ZP surrounds the larger human egg (120 μm diameter) and is composed of four secreted glycoproteins, ZP1 (528 aa, 100 kD), ZP2 (602 aa, 75 kD), ZP3 (328 aa, 55 kD) and ZP4 (444 aa, 65 kD) (Shabanowitz and O'Rand, 1988; Lefievre *et al*., 2004). In mice, each zona protein has a signal peptide to direct it into the secretory system with a transmembrane domain near the carboxyl terminus that tethers it to the endomembrane and similar motifs are present in the homologous human zona proteins (Ringuette *et al*., 1988; Chamberlin and Dean, 1990; Liang *et al*., 1990; Liang and Dean, 1993; Harris *et al*., 1994; Epifano *et al*., 1995; Boja *et al*., 2003; Lefievre *et al*., 2004). Within the endomembrane system, individual mouse zona proteins are glycosylated, mostly with *N*-glycans with a smaller mass of *O*-glycans (Tulsiani *et al*., 1992; Nagdas *et al*., 1994; Easton *et al*., 2000; Boja *et al*., 2003). After trafficking as individuals through the oocyte (Hoodbhoy *et al*., 2006; Jimenez-Movilla and Dean, 2010), each zona protein is cleaved upstream of a di-basic motif (Boja *et al*., 2003) and is released from the oocyte's plasma membrane to form the insoluble ZP (Jovine et al., 2002, 2004; Jimenez-Movilla and Dean, 2010).

The three secreted mouse proteins interact non-covalently to form the three-dimensional zona matrix. Each protein has a ZP domain of ~260 aa that contains eight conserved cysteine residues (Bork and Sander, 1992) and is divided into *N*-terminal 'ZP-N' and C-terminal 'ZP-C' subdomains. The zona domain, along with the cytoplasmic tails, control final assembly of the zona proteins into the extracellular matrix (Jovine *et al*., 2004; Han *et al*., 2010; Jimenez-Movilla and Dean, 2010). Upstream of the zona domain, a trefoil domain (http://pfam.sanger.ac.uk/family/trefoil) has been identified in ZP1 (Sommer *et al*., 1999) and ZP4 (Bork, 1993), but its function remains unknown. Intramolecular disulfide linkages stabilize the native conformation of the secreted zona glycoproteins, but only ZP1 has intermolecular disulfide bonds that form homodimers within the zona matrix (Bleil and Wassarman, 1980b; Epifano *et al*., 1995). Although the zona proteins have been well defined, including the three-dimensional structure of chicken ZP3 (Han *et al*., 2010), there remains considerable controversy over the nature of the zona ligand and the cognate sperm surface receptor necessary for gamete recognition.

Following fertilization, there is an effective block to polyspermy. First, within minutes, the egg plasma membrane is modified so that no additional sperm fuse with the egg. This block is not associated with changes in electrical potential observed in the other species, but its molecular basis remains unknown (Jaffe *et al*., 1983; Horvath *et al*., 1993). Also within minutes, additional sperm do not penetrate the ZP (Sato, 1979; Stewart-Savage and Bavister, 1988), but whether this reflects changes in the zona matrix or in sperm remains to be determined. Over several hours, the zona is further modified to preclude sperm binding. Although other changes have been inferred, the only documented biochemical modification of the ZP is the proteolytic cleavage of ZP2 after which the two fragments remain disulfide bonded (Bleil *et al*., 1981). This cleavage provides a definitive block to polyspermy by preventing sperm binding; if sperm do not bind, they cannot penetrate the zona matrix or fuse with the egg plasma membrane. However, prevention of post-fertilization ZP2 cleavage does not preclude live births, although decreased fecundity is observed (Gahlay *et al*., 2010; Burkart *et al*., 2012; Sachdev *et al*., 2012). Thus, the early blocks to polyspermy appear particularly important to ensure monospermic fertilization and the successful onset of development.

The puzzling role of ZP glycan ligands in gamete recognition

Using soluble, SDS-PAGE purified and re-natured zona proteins in an *in vitro* competitive sperm-binding assay with ovulated eggs, ZP3 initially was reported as the zona ligand for mouse sperm binding. No inhibitory effect of sperm binding to ovulated eggs was observed with re-natured ZP1 or ZP2, or with ZP3 isolated from the zona surrounding 2-cell embryos (Bleil and Wassarman, 1980a). These observations introduced the widely accepted glycan release model for gamete recognition which, with time, became increasingly precise to implicate *O*-glycans attached to Ser^{332} and Ser^{334} of ZP3 as zona ligands for sperm binding (Fig. 1). Following fertilization, these glycan ligands would be released by a putative glycosidase exocytosed from egg cortical granules to prevent sperm binding to 2-cell embryos (Florman and Wassarman, 1985; Chen *et al*., 1998). The nature of the carbohydrate ligand has remained controversial with α1,3 galactose and *N*-acetylglucosamine being proposed in mouse (Bleil and Wassarman, 1988; Miller *et al*., 1992) and the sialyl-$Lewis^X$ antigen in humans (Pang *et al*., 2011). However, mice lacking either the proposed zona ligand (α1,3 galactose) or the putative sperm receptor for *N*-acetylglucosamine are fertile (Thall *et al*., 1995; Asano *et al*., 1997; Lu and Shur, 1997). Furthermore, not only are Ser^{332} and Ser^{334} unadorned with carbohydrate in native mouse ZP (Boja *et al*., 2003), but mutating the sites to prevent glycosylation does not adversely affect fertility in transgenic mice (Liu *et al*., 1995), even in the absence of endogenous normal ZP3 (Gahlay *et al*., 2010).

An additional complexity for glycan-release models is the heterogeneity of zona glycans, which implies a need for a range of different sperm surface receptors or that only subsets of zona ligands are biologically active. The appeal of glycan-release models is also diminished with continued fertility of mice lacking specific glycosyl transferases which restricts the pool of possible glycan ligands (Asano *et al*., 1997; Lu and Shur, 1997; Lowe and Marth, 2003; Shi *et al*., 2004;

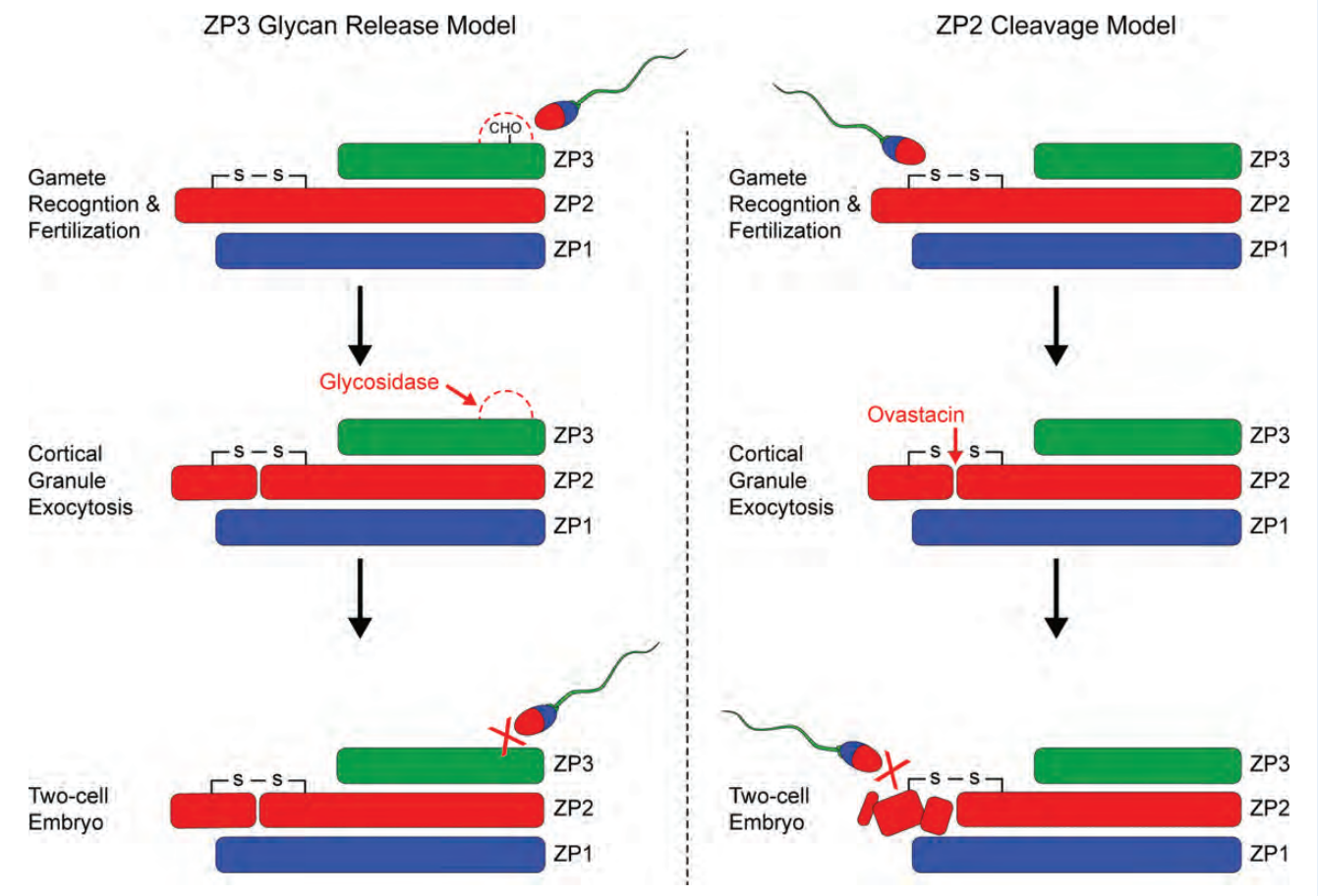

Figure 1 Models of gamete recognition. A specific glycan-release model (left panel) proposes that *O*-glycans on ZP3 Ser^{332} and Ser^{334} act as ligands for a sperm surface receptor. Following fertilization, cortical granules would release a glycosidase that would remove the *O*-glycans and account for the inability of sperm to bind to 2-cell embryos. The ZP2 cleavage model (right panel) proposes that sperm bind to an *N*-terminal domain of ZP2. Following fertilization, ovastacin (encoded by *Astl*), a cortical granule metalloendoprotease is exocytosed and the proteolytic destruction of the ZP2 docking site prevents sperm from binding to 2-cell embryos. Each model has been tested using mouse transgenesis. Mouse zonae pellucidae containing human rather than mouse ZP3 do not support human sperm binding and $Zp3^{S332A;S334A}$ mutant mice have normal fertility. These results are not consistent with the ZP3 glycan-release model as currently articulated. In contrast, mouse zonae pellucidae containing human rather than mouse ZP2 support human sperm binding/penetration and $Zp2^{Mut}$ and $Astl^{Null}$ mice in which ZP2 remains uncleaved in 2-cell embryos support *de novo* sperm binding despite fertilization and cortical granule exocytosis. These results support the ZP2 cleavage model of gamete recognition (Gahlay *et al*., 2010; Baibakov *et al*., 2012; Burkart *et al*., 2012).

Williams *et al*., 2007). More recently, it has been reported that if ZP2 remains intact, sperm bind to the ZP independent of fertilization and cortical granule exocytosis (Rankin *et al*., 2003; Baibakov *et al*., 2007; Gahlay *et al*., 2010; Burkart *et al*., 2012). This observation is not consistent with the post-fertilization exocytosis of a cortical granule glycosidase that would prevent sperm binding by release of a ligand, an axiom integral to the glycan-release model. For sperm to bind to the ZP after fertilization and cortical granule exocytosis, the candidate glycan would have to remain accessible for sperm binding and yet have been inaccessible for cleavage by a cortical granule glycosidase.

The cause of the discrepancy between the initial biochemical- and more recent genetic-based assays in the assessment of the ZP3 glycan-release models remains unclear. It may be that soluble zona proteins isolated by SDS-PAGE and then re-natured do not provide a realistic proxy for the same protein in the insoluble ZP. Of note, these assays never achieved 100% inhibition of sperm binding and only one sperm is necessary for fertilization. Alternatively, it has been reported that solubilized ZP3 triggers the acrosome reaction and acrosome-reacted sperm do not bind to the ZP (Saling *et al*., 1979). However, this possibility was considered less likely (Bleil and Wassarman, 1980a, 1983) and would not explain the observation that ZP3 glycopeptides inhibit sperm binding (Florman and Wassarman, 1985), but do not induce the acrosome reaction (Florman *et al*., 1984; Leyton and Saling, 1989). Because of these aforementioned experimental results and caveats, other models of sperm–egg recognition have been sought.

The ZP2 cleavage model of gamete recognition

Mouse genetics has been used to systematically test the role of individual zona proteins in gamete recognition. Each single-copy mouse gene

has been successfully targeted in embryonic stem cells which were then used to establish mouse lines lacking ZP1, ZP2 or ZP3. In the absence of ZP1, mice form a more loosely woven zona matrix, but remain fertile albeit with decreased fecundity (Rankin *et al*., 1999). A more dramatic phenotype is observed in the absence of either ZP2 or ZP3 (Liu *et al*., 1996; Rankin *et al*., 1996, 2001). In both instances, there is no ZP surrounding ovulated eggs which are quickly resorbed into the epithelial lining of the oviduct, a phenomenon previously reported following biochemical removal of the zona matrix (Bronson and McLaren, 1970; Modlinski, 1970). Both $Zp3^{Null}$ and $Zp3^{Null}$ mouse lines are uniformly sterile. Thus, ZP1 is not essential for gamete recognition and fertility, but the absence of a ZP matrix in the null mice precludes the assessment of either ZP2 or ZP3 in gamete recognition.

Eutherian mammals have syntenic loci encoding single copies of ZP1, ZP2, ZP3 and ZP4 (Spargo and Hope, 2003; Boja *et al*., 2005; Goudet *et al*., 2008), although no mouse ZP4 protein is present because of multiple stop and missense codons (Lefievre *et al*., 2004). The primary structure of secreted mouse and human ZP1 (71%), ZP2 (62%) and ZP3 (71%) are well conserved, but gamete recognition is relatively taxon-specific and human sperm will not bind to the mouse ZP (Bedford, 1977) nor fuse with mouse eggs (Quinn, 1979; Yanagimachi, 1984). Therefore, to investigate further the roles of ZP2 and ZP3, transgenic mouse lines in which human ZP1, ZP2 and ZP3 replaced the endogenous mouse proteins were established and human sperm were used to interrogate the human–mouse chimeric zonae pellucidae. Human sperm bound to the ZP in the presence of human ZP2, but not in the presence of human ZP1, ZP3 or ZP4 (Yauger *et al*., 2011; Baibakov *et al*., 2012). This was a dominant genetic effect and required that human sperm be capacitated to bind to the surface of the ZP (Fig. 2). In mice with a ZP formed by four human proteins and lacking the three endogenous mouse proteins, human sperm bound and penetrated the zona matrix. Acrosome-reacted human sperm accumulated in the perivitelline space unable to fuse or fertilize mouse eggs (Baibakov *et al*., 2012). Taken together, it appears that ZP2 rather than ZP3 is the zona ligand to which human sperm bind.

Following fertilization, ZP2 is cleaved and sperm do not bind to 2-cell mouse embryos (Bleil *et al*., 1981). The ZP2 cleavage site was defined biochemically (^{166}LA$^{\downarrow}$DE^{169}) as that recognized by the astacin family of metalloendoproteases (Gahlay *et al*., 2010). Ovastacin, an oocyte-specific astacin (Quesada *et al*., 2004) encoded by *Astl*, is released from cortical granules and is responsible for the post-fertilization cleavage of ZP2 (Burkart *et al*., 2012). Mutation of the cleavage site or ablation of the gene encoding ovastacin leaves ZP2 intact following fertilization and supports sperm binding to the ZP despite cortical granule exocytosis (Gahlay *et al*., 2010; Burkart *et al*., 2012) (Fig. 3). Using truncated recombinant ZP2 peptides in a bead-binding assay, the sperm-binding site was further refined to ~115 aa at the *N*-terminus of ZP2 (Baibakov *et al*., 2012).

These observations led to a simple, unifying formulation for the molecular basis of gamete recognition in which capacitated sperm attach to an *N*-terminal domain of ZP2 prior to zona penetration and gamete fusion. Following fertilization, ovastacin is released from egg cortical granules and cleaves extracellular ZP2 (Burkart *et al*., 2012). This effectively destroys the sperm-binding domain (Greenhouse *et al*., 1999; Gahlay *et al*., 2010; Burkart *et al*., 2012) and accounts for the inability of sperm to bind to 2-cell embryos (Fig. 1). There is a single *N*-glycan at Asn^{83}, but no *O*-glycans in this region of native mouse ZP2$^{35-149}$ (Boja *et al*., 2003), but whether or not this glycan plays a role in gamete recognition has not been determined.

Although earlier reports implicated ZP2 as a secondary ligand for acrosome-reacted sperm (Bleil *et al*., 1988; Tsubamoto *et al*., 1999; Kerr *et al*., 2002; Chakravarty *et al*., 2008; Chiu *et al*., 2008), the *Xenopus laevis* homolog, gp69/64, inhibits primary sperm binding to eggs *in vitro* (Tian *et al*., 1997). Following fertilization and cleavage at the conserved ^{155}FD$^{\downarrow}$DD^{158} site, the C-terminal native gp69/64 glycopeptide does not affect sperm binding. Although a short recombinant peptide gp69/64$^{130-156}$ did not inhibit sperm binding, the longer gp69/64$^{34-156}$ *N*-terminal domain (homologous to mouse ZP2$^{35-149}$) was not tested. Following fertilization *Xenopus laevis* ZP2 is cleaved by a zinc metalloprotease (Lindsay and Hedrick, 2004) and post-fertilization proteolysis of the *N*-terminal domain of gp69/64 could account for the inability of sperm to bind to early *Xenopus laevis* embryos as it does in mice. Thus, the ZP2 cleavage model of gamete recognition may pertain more broadly among vertebrates.

Sperm receptor candidates mediating fertilization

It has long been presumed that gamete recognition of a ZP ligand requires a cognate sperm receptor, but its identity and localization have defied definition. The acrosome is a subcellular organelle that underlies the anterior surface of the sperm. During fertilization, its outer membrane fuses with the plasma membrane releasing macromolecules, the functions of which are incompletely understood. Although the sperm receptor traditionally has been considered to reside in the plasma membrane, acrosome-reacted sperm lacking the anterior plasma membrane are reported to fertilize eggs *in vitro* (Fleming and Yanagimachi, 1982; Kuzan *et al*., 1984; Inoue *et al*., 2011; Jin *et al*., 2011) and the site of acrosome exocytosis remains under active investigation (Avella and Dean, 2011). There is a surprising surfeit of sperm surface receptor candidates for the seemingly simple organization of the mammalian ZP. Several strong candidates have arisen from biochemical and cell biological analyses that documented their importance in gamete recognition using *in vitro* assays. Unexpectedly, genetic ablation of individual candidates in transgenic mice did not prevent fertility. This suggests that the *in vitro* assays used to support their candidacy may not have captured the physiological complexity inherent in *in vivo* gamete recognition. Alternatively, it may be that multiple gene products are required to form a sperm surface complex that interacts with the ligand in the ZP *in vivo*. However, it is not clear why a need for additional proteins would not have been noted in the *in vitro* assays initially used to propose the candidate protein. Redundancy of sperm surface receptors has also been invoked to account for the difficulty in forming a consensus on the correct sperm surface receptor. Here we briefly review sperm surface candidates that have been tested by genetic ablation of the cognate gene in transgenic mice (Table I).

Sperm surface receptor candidates for binding to the ZP

β1,4-galactosyltransferase (GalTase) was one of the first sperm surface molecule proposed to play a role in sperm–egg binding.

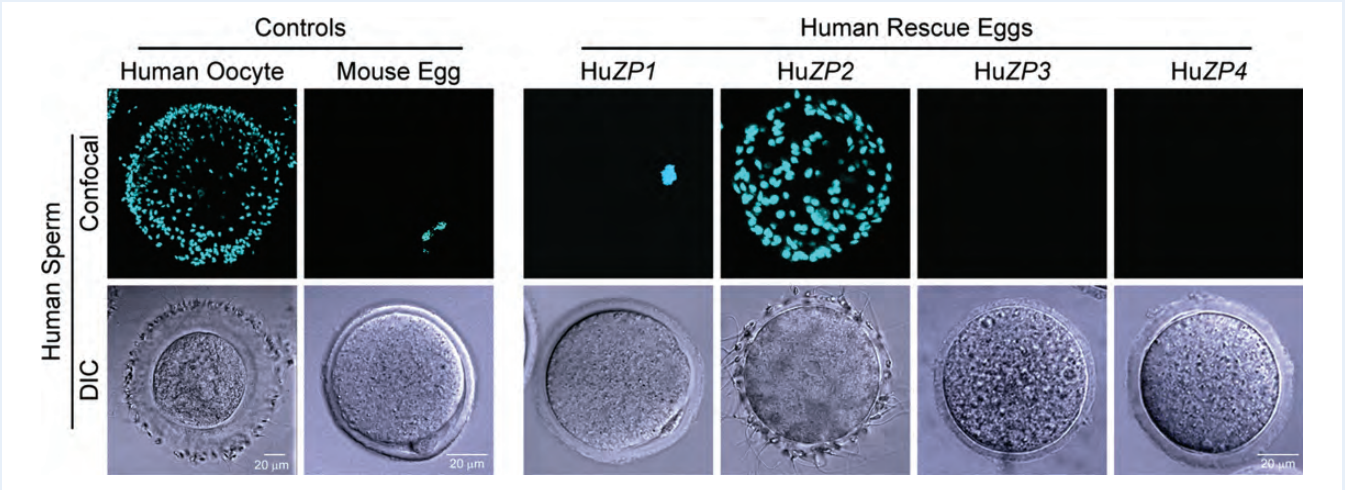

Figure 2 Human sperm bind to transgenic mice expressing human ZP2 in the ZP. Confocal and differential interference contrast images of capacitated human sperm binding to the ZP of mice expressing human ZP1, human ZP2, human ZP3 or human ZP4, in the absence of the corresponding mouse protein. Human oocytes and mouse eggs serve as positive and negative controls, respectively. Modified from ref. Baibakov *et al.* (2012).

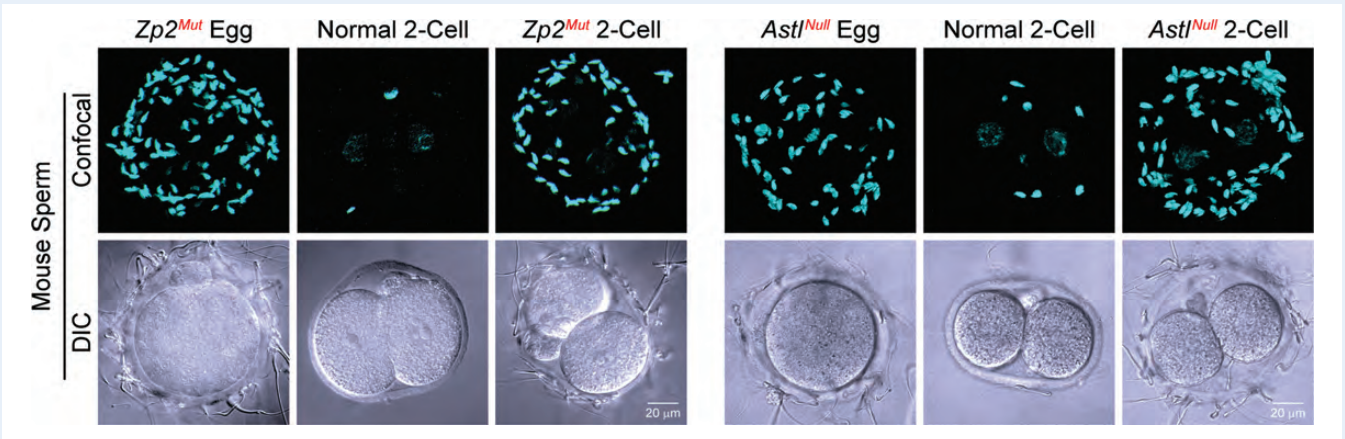

Figure 3 Mouse sperm bind to 2-cell embryos, if ZP2 remains uncleaved. Capacitated mouse sperm binding to 2-cell embryos in which ZP2 remains intact because ZP2 was mutated to prevent post-fertilization cleavage ($Zp2^{Mut}$, left panel) or because of the absence of ovastacin ($Astl^{Null}$, right panel). $Astl^{Null}$ and $Zp2^{Mut}$ eggs serve as positive controls and normal 2-cell embryos as negative controls. Modified from refs Gahlay *et al.* (2010) and Burkart *et al.* (2012).

Male mice with mutant alleles of the *T/t* complex exhibit transmission distortion ratios in which the mutant *t*-allele is preferentially passed to the next generation (Fraser and Dudley, 1999). Based on a reported 4-fold increase of enzymatic activity in the mutant allele, a role was proposed for GalTase in gamete recognition (Shur, 1981). GalTase is a member of the galactosyltransferase superfamily of enzymes that are responsible for the synthesis of glycoside residues of glycoproteins, glycolipids and glycosaminoglycans (Joziasse, 1992). GalTase is localized in the endoplasmic reticulum, the Golgi apparatus and on the cell surface of a variety of cells. It has been proposed to mediate cell–cell interactions by binding to the glycoconjugate substrates on the adjacent cell surface and extracellular matrix (Roseman, 1970; Hathaway and Shur, 1988). GalTase was reported to interact directly with O-linked oligosaccharides on ZP3 (Miller *et al.*, 1992) and inhibiting GalTase activity or blocking its recognition site on the ZP greatly diminished sperm binding (Shur and Hall, 1982). Both affinity-purified GalTase and anti-GalTase antibodies disrupted sperm–zona binding in a dose-dependent manner (Lopez *et al.*, 1985). However, genetic ablation of GalTase did not significantly affect male fertility (Asano *et al.*, 1997; Lu and Shur, 1997).

A second candidate sperm receptor, ZP3R (sp56), was initially localized on the plasma membrane of acrosome-intact mouse sperm (Cheng *et al.*, 1994; Suzuki-Toyota *et al.*, 1995). ZP3R was reported to possess affinity for ZP3 glycans (Bleil and Wassarman, 1990; Cheng *et al.*, 1994). Purified native or recombinant ZP3R inhibited sperm binding to the ZP surrounding mouse eggs, but not to embryos *in vitro* (Bookbinder *et al.*, 1995; Buffone *et al.*, 2008). Subsequent studies relocated ZP3R within the acrosomal matrix (Foster

Table I Sperm receptor candidates for zona binding ablated in transgenic mice.

Sperm protein	Gene	Expression	Localization	Zona ligand	Null phenotype
GalTase: UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 1	*B4galt1*	Ubiquitous	Plasma membrane	ZP3	Fertile
SED1 (MFG-E8): milk fat globule-EGF factor 8	*Mfge8*	Ubiquitous	Plasma membrane	ZP3, ZP2	Subfertile or fertile
SPAM1 (PH20): sperm adhesion molecule 1	*Spam1*	Testis specific	Inner acrosomal membrane	Not specified	Fertile
ZP3R (SP56): ZP 3 receptor	*Zp3r*	Testis specific	Acrosomal matrix	ZP3	Fertile
Proacrosin acrosin prepropeptide	*Acr*	Testis specific	Acrosomal matrix	ZP2	Fertile
Zonadhesin	*Zan*	Testis specific	Outer acrosomal membrane	Not specified	Fertile
ADAM3 (cyritestin): a disintegrin and metallopeptidase domain 3	*Adam3*	Testis specific	Plasma membrane	Not specified	Sterile

et al., 1997; Kim et al., 2001) and genetic ablation of the cognate gene did not affect male fertility (Muro et al., 2012).

Using native, particulate pig ZP as an affinity matrix, two proteins were isolated from pig sperm membranes and identified as candidate proteins for adherence of sperm to the ZP. One was the aforementioned ZP3R and the other was designated zonadhesin (Hardy and Garbers, 1994, 1995). Zonadhesin is expressed uniquely in the testes and contains multiple cell adhesion molecule domains, including MAM (meprin/A5 antigen/mu receptor tyrosine phosphatase), mucin and von Willebrand D domains (Hardy and Garbers, 1995; Gao and Garbers, 1998). Initially present in the outer acrosomal membrane and outer aspect of the acrosomal matrix (Bi et al., 2003), zonadhesin becomes exposed on the sperm surface during capacitation (Tardif et al., 2010). As with other candidates, native isoforms and recombinant zonadhesin bound to the ZP (Hickox et al., 2001; Lea et al., 2001) and anti-zonadhesin antibody significantly reduced sperm binding and IVF (Tardif et al., 2010). However, zonadhesin null males were fertile (Tardif et al., 2010).

SED1 (secreted protein that contains notch-like epidermal growth factor (EGF) repeats and discoidin/F5/8 type C domains) is the mouse homolog of a boar sperm surface protein (p47) isolated by affinity chromatography with porcine zona proteins (Ensslin et al., 1995, 1998). Mouse SED1 is secreted by the epididymal epithelium and attaches to the sperm plasma membrane overlying the acrosome when sperm progress through the epididymis. SED1 binds to ZP3 on immunoblots and to a lesser extent to ZP2 (Ensslin and Shur, 2003). The protein is encoded by *Mfge8* (milk fat globule-EGF 8) and has been genetically ablated by four groups of investigators. In each case, male mice were fertile, with either decreased (Ensslin and Shur, 2003; Silvestre et al., 2005) or normal (Hanayama et al., 2004; Neutzner et al., 2007) fecundity.

Among the field of sperm receptor candidates, ADAM3 (a disintegrin and metalloprotease 3) is noteworthy in providing an infertile phenotype in mice lacking the protein (Shamsadin et al., 1999; Nishimura et al., 2001). In addition, multiple genes including *Ace*, *Clgn*, *Adam2*, *Adam1a*, *Calr3*, *Tpst2*, *Rnasse10*, *Pdilt* and *Pmis2*, when disrupted, display defective zona-binding ability to cumulus-free eggs and impaired migration into the oviduct similar to that observed in *Adam3* null males (Krege et al., 1995; Ikawa et al., 1997, 2011; Cho et al., 1998; Nishimura et al., 2004; Marcello et al., 2011; Krutskikh et al., 2012; Tokuhiro et al., 2012; Yamaguchi et al., 2012). All of these additional null mutations lack ADAM3 which suggests a common denominator for their observed phenotype. However, mutant sperm lacking ADAM3 fertilized eggs *in vivo* following injection into the oviduct and *in vitro*, in the presence, but not in the absence, of the cumulus oophorus (Tokuhiro et al., 2012; Yamaguchi et al., 2012). Thus, rather than gamete recognition, the major role for ADAM3 may be in mediating passage of sperm through the utero-tubal junction. Of note, the orthologous gene in humans is two non-functional pseudogenes (Grzmil, 2001) and men are fertile in the absence of ADAM3 protein.

Acrosome-reacted sperm receptor candidates for binding to the ZP

A second group of sperm receptor candidates has been reported to interact with the ZP after sperm acrosome exocytosis. PH20, later renamed SPAM1 (sperm adhesion molecule 1), is a glycosyl phosphatidylinositol-anchored protein first identified in guinea pigs. This protein is located on both the plasma membrane and the inner acrosomal membrane of the sperm head and contains a ZP binding domain near the C-terminus and a hyaluronidase domain on the *N*-terminus with enzymatic activity (Hunnicutt et al., 1996). Monoclonal antibodies to SPAM1 inhibited the binding of acrosome-reacted, but not acrosome intact, guinea pig sperm to the ZP (Primakoff et al., 1985; Myles et al., 1987) and immunization resulted in 100% effective, but reversible contraception (Primakoff et al., 1988). Although native and recombinant SPAM1 have hyaluronidase activity (Gmachl et al., 1993; Lin et al., 1994; Hunnicutt et al., 1996), *Spam1* null male mice were fertile. Mouse sperm lacking SPAM1 penetrated, albeit with decreased efficiency, the hyaluronan of the cumulus oophorus and fertilized eggs *in vitro* (Baba et al., 2002).

Acrosin, another well-investigated sperm molecule implicated in sperm–zona interaction, is present ubiquitously in the acrosome matrix. It was initially detected in detergent extracts of boar sperm as a binding partner of the pig ZP (Brown and Jones, 1987; Jones and Brown, 1987; Topfer-Petersen and Henschen, 1987) and subsequently identified as proacrosin (Jones, 1991; Urch and Patel, 1991; Richardson and O'Rand, 1996). Cleavage of proacrosin activates acrosin, a major serine protease of mouse sperm (Stambaugh and Buckley, 1972; Huang-Yang and Meizel, 1975; Baba et al., 1989). Due to its potential roles in zona binding and proteolysis, acrosin

was inferred to play a role in gamete recognition and zona matrix penetration (Stambaugh and Buckley, 1972; Topfer-Petersen and Henschen, 1987, 1988). Although acrosin exhibited an affinity with ZP2 (Urch and Patel, 1991; Howes *et al*., 2001) and may convey a selective advantage to sperm, male mice lacking acrosin remained fertile (Baba *et al*., 1994; Yamagata *et al*., 1998).

The difficulty in identifying a sperm surface protein as essential for gamete recognition has been puzzling. One heretical thought is that sperm might not need to bind to the ZP prior to penetration and fusion with eggs. This might account for observations that acrosome-reacted sperm can penetrate and fertilize zona-intact eggs *in vitro* (Fleming and Yanagimachi, 1982; Kuzan *et al*., 1984; Inoue *et al*., 2011; Jin *et al*., 2011). However, a non-binding paradigm for gamete recognition would be difficult to reconcile with the observed taxon specificity of gamete recognition reported *in vitro* (Baibakov *et al*., 2012). Thus, the search for the elusive sperm surface protein involved in gamete recognition continues.

Perspectives

Mouse genetics have proved useful in testing established models of gamete recognition and in proposing new paradigms for further investigation. The widely accepted ZP3 glycan-release models first introduced more than 30 years ago are not consistent with recent results in transgenic mice. However, the newly introduced ZP2 cleavage model of gamete recognition is supported by the taxon-specificity of human sperm binding to zonae pellucidae in which human ZP2 replaces endogenous mouse ZP2 and the discovery of ovastacin as the cortical granule metalloendoprotease that cleaves ZP2 to prevent post-fertilization sperm binding. This paradigm-shifting model is falsifiable and predicts that human sperm will bind to mouse ZP2 containing a human ZP2 *N*-terminal domain and that female mice lacking the mouse ZP2 *N*-terminal domain will be sterile. Both predictions can be evaluated in transgenic mice expressing the appropriate mutant form of ZP2 in the *Zp2* null background. However, identification of the zona ligand is only half the story for understanding the molecular basis of gamete recognition at the surface of the ZP.

If prior focus on ZP3 was misplaced, the current molecular definition of ZP2 as the zona ligand offers a path forward in biochemically identifying the cognate sperm surface receptor. Once nominated, an essential sperm receptor candidate should meet particular criteria including *in vivo* and *in vitro* infertility after genetic ablation in transgenic mice. This phenotype should be rescued by expressing the human orthologue in transgenic mice and crossing them into the *Zp2* null background so that they express only the human sperm receptor. Sperm from these mice should bind and penetrate the zonae pellucidae containing human ZP2. If fertile, these mice would provide compelling evidence for the candidacy of the identified sperm receptor. Conversely, taxon-specificity should be preserved and the 'humanized' transgenic sperm should not bind or fertilize normal mouse eggs. We note that the putative sperm receptor need not be a single protein, but could be a complex which would significantly complicate its identification.

A 'humanized' model with male mice expressing the human sperm receptor and female mice with human ZP2 in their ZP would establish a robust system for further investigation of human gamete interactions. For example, the ability of human sperm to bind to the 'humanized' ZP could prove useful in identifying human sperm most suitable for ICSI and contraceptive strategies aimed at pre-fertilization cleavage of ZP2 to prevent sperm binding to ovulated eggs could be experimentally addressed in this model system. While significant progress has been made in our understanding of the molecular basis of fertilization and the post-fertilization block to polyspermy, many investigative challenges remain.

As the field progresses in providing a detailed explanation of gamete recognition, these advances should rapidly empower reproductive medicine and inform on the diverse etiologies of human infertility as well as provide improved strategies for family planning.

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Authors' roles

M.A.A. and B.X. wrote the manuscript which was edited by J.D. who also contributed to the figures.

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Conflict of interest

None declared.

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