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Review

A role for carbohydrate recognition in mammalian sperm-egg binding

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ABSTRACT

Mammalian fertilization usually requires three sequential cell–cell interactions: (i) initial binding of sperm to the specialized extracellular matrix coating the egg known as the zona pellucida (ZP); (ii) binding of sperm to the ZP via the inner acrosomal membrane that is exposed following the induction of acrosomal exocytosis; and (iii) adhesion of acrosome-reacted sperm to the plasma membrane of the egg cell, enabling subsequent fusion of these gametes. The focus of this review is on the initial binding of intact sperm to the mammalian ZP. Evidence collected over the past fifty years has confirmed that this interaction relies primarily on the recognition of carbohydrate sequences presented on the ZP by lectin-like egg binding proteins located on the plasma membrane of sperm. There is also evidence that the same carbohydrate sequences that mediate binding also function as ligands for lectins on lymphocytes that can inactivate immune responses, likely protecting the egg and the developing embryo up to the stage of blastocyst hatching. The literature related to initial sperm-ZP binding in the three major mammalian models (human, mouse and pig) is discussed. Historical perspectives and future directions for research related to this aspect of gamete adhesion are also presented.

Contents

1. Introduction

The very first cell–cell interaction in the life of sexually reproducing metazoans is the binding of sperm to the surface of the egg. The matrix covering the egg is highly specialized to ensure that robust binding occurs, initiating the process of fertilization. Rapid species-specific binding is also obligatory during external fertilization in an aquatic environment, especially in the presence of closely related species within the same ecological niche [1]. There are many barriers that block reproduction between different mammalian species, so the requirements for species-specific binding are more relaxed [2,3]. A notable exception is the restricted specificity of human sperm that bind only to the zona pellucida (ZP) of hominids like the gorilla and gibbon [3,4].

The concept that sperm-egg binding involves carbohydrate recognition was initially proposed by Monroy nearly fifty years ago [5]. This specificity was based on the sensitivity of gamete binding to mild periodate oxidation of the jelly coat encasing eggs in lower

Abbreviations: ZP, zona pellucida; NK, natural killer; SRS, species recognition system; EBP, egg binding protein; SLEX, sialyl-Lewis^x; MS, mass spectrometry; HZA, hemizona assay; Cer, ceramide.

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marine species. Sodium *m*-periodate under carefully controlled conditions selectively oxidizes vicinal hydroxyl groups associated with oligosaccharides and polysaccharides coating the surface of eggs [6]. Sperm-egg binding was the first cell–cell interaction that was clearly shown to depend on carbohydrate recognition based on this specific chemical sensitivity.

Propagation is an absolute imperative for the survival of any species. In higher organisms, a functional immune response that targets pathogens and tumor cells is also essential for individuals to survive long enough to generate fertile progeny. These overlapping imperatives suggest that the immune and reproductive system must be highly integrated to enable such responses against pathogens while simultaneously protecting gametes and the developing fetus in utero from injury. Immune cells usually recognize cells, tissues and organs via their major histocompatibility (MHC) class I antigens [7]. This recognition of self in the immunological context is necessary to prevent aberrant responses against healthy cells and tissues while enabling highly specific targeting of pathogens and tumor cells. However, gametes and many other cell types in the human body completely lack MHC antigens, or express these molecules at a very low level [8]. This lack of MHC expression places these cells at risk for lysis by natural killer (NK) cells based on the missing self hypothesis [9].

A species recognition system (SRS) has previously been hypothesized to prevent the lysis of MHC class I negative cell types [10]. The SRS relies on a system of lectins bearing domains that inhibit immune responses (e.g. immunoreceptor tyrosine-based inhibition motifs or ITIM) or that can form a complex with a protein possessing immune-modulating activities. By working together, the SRS and the MHC system enable immune recognition of all types of cells and tissues in mammals. This SRS is also employed during initial sperm-egg binding during the process of fertilization [10]. For this reason, the SRS likely predates the MHC system for the recognition of self, which developed later to enable more precise targeting of pathogens and tumor cells. The potential linkages between gamete and immune recognition in the context of the SRS have previously been reviewed [11].

The evidence that initial sperm-ZP binding in the human, mouse and pig relies primarily on carbohydrate recognition is now quite substantial. The primary focus of this review is to emphasize the implications of this specificity of binding and discuss future directions.

2. Human sperm-ZP binding

For many years, the mouse was the predominant model for mammalian gamete binding interactions. The assumption was that data

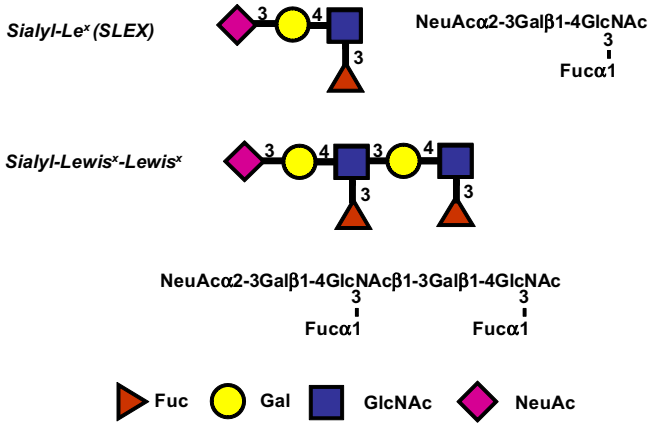

Fig. 1. Major terminal carbohydrate sequences expressed on the surface of the human ZP.

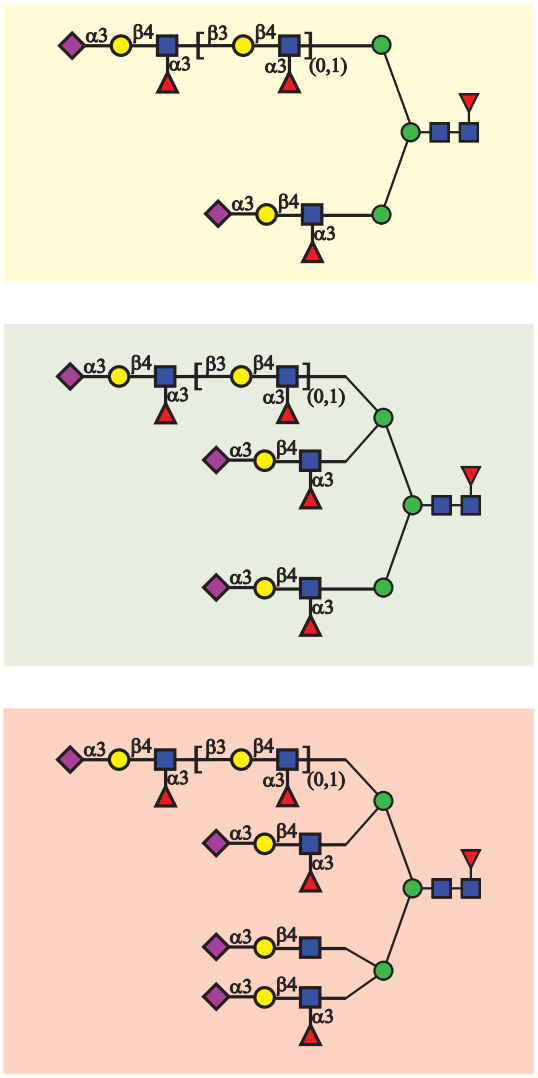

Fig. 2. Major N-linked glycans expressed on the surface of the human ZP. This figure was previously published [14].

obtained in the mouse model could be directly extrapolated to the human model, but recent studies indicate otherwise. The human has now become the predominant model for investigating sperm-ZP binding interactions that have translational value in the clinical setting. A definitive review on the role of carbohydrate recognition in human sperm-ZP binding, including a brief historical perspective, has recently been published [12]. Another review focused on the identification of the egg binding proteins (EBPs) associated with human and mammalian sperm is also available [13].

The most definitive study on human sperm-ZP binding was published in 2011 [14]. The human ZP is profusely coated with carbohydrate sequences known as sialyl-Lewis^x (SLEX) and sialyl-Lewis^x-Lewis^x (Fig. 1). A summary of the major types of the core fucosylated N-glycans associated with human ZP is also shown (Fig. 2). Terminal SLEX is expressed on about 85% of all the N-glycans, usually in multivalent presentations. SLEX tetrasaccharide and SLEX-BSA neoglycoprotein inhibit human sperm-ZP binding in the hemizona assay (HZA) by 70% at final concentrations of 500 μM and 2 μM, respectively [14]. These findings are consistent with a previous study indicating that 79% of human sperm binding in the HZA relies on lectin-like interactions, with protein–protein interactions responsible for the remaining activity [15]. SLEX is also a ligand for all three selectins and Siglec-9, consistent with the SRS paradigm [16–18].

Another striking feature of the N-glycan profile of human ZP is the complete absence of any high mannose type N-glycans. This presentation is in sharp contrast to the N- and O-glycans associated with murine ZP [19–22]. Definitive analysis of murine ZP glycosylation reveals substantial amounts of high mannose type N-glycans and a far greater structural diversity of complex type N- and O-glycans compared to human ZP glycans. Similar structural diversity of N- and O-glycan sequences is also observed in porcine ZP glycoproteins, although no high mannose N-glycans are present in this matrix [23–25]. The relatively simple pattern of N- and O-glycans associated with the human ZP suggests a much more highly focused glycosylation process that results in the abundant presentation of SLEX. This result suggests that SLEX could also be employed to mediate the binding of acrosome-reacted sperm to the ZP.

This restriction of ZP glycosylation in the human versus the mouse and pig suggests that the regulation of glycosylation could be positively correlated with the degree of organismal complexity. Evidence favoring this concept would require the sequencing of ZP glycoproteins from other mammals at different levels of the evolutionary ladder. Nonetheless, the highly restricted presentation of SLEX and sialyl-Lewis^x-Lewis^x on the human ZP is consistent with the concept that glycans in some cases act as functional groups for critical physiological activities, i.e., functional glycosylation.

Restricted glycan expression is also observed in human leukocyte antigen (HLA) class I molecules, where unusual uniformity of N-glycan sequences has been documented [26]. Another notable example is the addition of unusual O-mannosylated glycans to dystroglycan and other proteins. Aberrant expression of these mannosylated glycans leads to the development of different pathological states, including congenital forms of muscular dystrophy [27]. There are other examples, and it is likely that additional evidence confirming that glycans act as functional groups will emerge as more glycoproteins are subjected to glycomic analysis.

Many different EBPs have been proposed to mediate human sperm-ZP binding (Table 1) [28–38]. Most of these potential EBPs were proposed years before the glycans linked to the human ZP were finally sequenced [14]. There is now near universal acceptance that human sperm-egg binding relies primarily on the recognition of terminal SLEX. These results indicate that only native human ZP or recombinant human ZP glycoproteins bearing N- and O-glycans terminated with SLEX should be employed to define EBPs. Studies that do not adhere to this requirement are far less likely to be physiologically relevant. Thus far, only fucosyltransferase V (FUT5) and hCRISP1 have been shown to bind to native human ZP [34,35]. Since hCRISP1 also binds to recombinant ZP3 synthesized in an insect baculovirus system, it is unlikely that this potential EBP mediates binding solely via lectin-like interactions involving SLEX [35]. No putative EBP has yet been tested on a glycan microarray to determine if it binds to SLEX [39].

Table 1
Proposed human egg binding proteins.

Adhesion molecule	References
PH-20	[28]
α-Mannosidase	[29]
Mannose binding protein	[30]
FA-1	[31]
Selectin-like molecule	[32]
α-Fucosidase	[33]
α1–3 Fucosyltransferase	[34]
hCRISP1	[35]
Voltage dependent anion channel 2	[36]
Glutathione reductase	[36]
Human dicarbonyl/L-xylulose reductase (DCXR)	[37]
Heat shock protein A2 complex	[38]

3. Murine sperm-ZP binding

Until recently, the mouse was the predominant model for mammalian sperm-egg binding. This preeminence is reflected in numerous reviews on this topic published since 1983, only a few of which are referenced here [40–46]. The major reasons for employing this animal model include its small size, brief lifespan, fertility, established genetics and utility for genomic manipulation.

The murine ZP consists of three major glycoproteins known as ZP1, ZP2 and ZP3 [47]. ZP3 mediates both sperm binding and evokes acrosomal exocytosis [48]. Deletion of ZP3 causes a complete loss of ZP and fecundity [49]. The biological activity of ZP3 remains after protein denaturation, suggesting that its oligosaccharides likely mediate binding [50]. Murine sperm-egg binding is also blocked by either pronase glycopeptides or O-glycans isolated from ZP3 [50,51]. Treatment of ZP3 with α-galactosidase inactivates the ability of O-glycans to block murine sperm-egg binding, indicating that terminal α-linked galactose is essential for its sperm binding activity [52].

Sequencing of mouse ZP N-glycans reveals the presence of high mannose and complex type biantennary, triantennary, and tetraantennary N-glycans [20–22]. The terminal ends of these N-glycans include lacNAc (Galβ1–4GlcNAc), sialylated lacNAc (NeuAcα2–3Galβ1–4GlcNAc, NeuGcα2–3Galβ1–4GlcNAc), Sd^a antigen (NeuAcα2–3[GalNAcβ1–4]Gal, NeuGcα2–3[GalNAcβ1–4]Gal), and terminal GlcNAc. In some cases, these sequences cap the terminal ends of polylactosamine extensions. Core 2 sequences dominate the O-glycan pool, where they are terminated with sialic acid, lacdiNAc (GalNAcβ1–4GlcNAc), α-galactosylated lacNAc (Galα1–3Galβ1–4GlcNAc) and the Sd^a antigen terminated with NeuAc or NeuGc [19].

Recombinant ZP3 synthesized in F9 embryonal carcinoma cells is as biologically active as native ZP3 [53]. Subsequent genetic manipulations indicated that a region of ZP3 (Ser-329 to Ser-334; SNSSSS) is necessary for sperm binding [54]. The substitution of Ser-332 or Ser-334 with alanine yields biologically inactive recombinant ZP3 [55]. This result indicated that O-glycans positioned at these serine residues are required for sperm binding. An EBP implicated in binding designated sp56 was also identified [56].

Another research group proposed that a specific β1–4 galactosyltransferase expressed on the sperm head region is the major murine EBP [57]. This glycosyltransferase was subsequently postulated to bind to terminal GlcNAc residues on O-linked polylactosaminoglycans linked specifically to ZP3 [58]. In this model, polyspermy is inhibited by the release of a specific β-N-acetylglucosaminidase during the cortical reaction, which then cleaved off these terminal GlcNAc residues [59].

Subsequent experiments did not support either of these two major models for murine sperm-ZP binding. Female mice lacking terminal α-linked galactose due to a specific glycosyltransferase knockout remain fertile [60]. Their eggs bind sperm as well as eggs from wild type mice [61]. O-glycans are not linked to Ser-332 and Ser-334 in native ZP3 based on the results of MS analyses [62,63]. sp56 is located in the acrosome and not the plasma membrane, where it must be positioned to mediate sperm binding to the ZP [64,65]. Mutant mice lacking the sperm surface β-galactosyltransferase are as fertile as wild type mice and their sperm bind to eggs in appreciably higher levels (3–4-fold) than sperm derived from wild type mice [66]. Ultrasensitive MS methods do not detect any O-linked polylactosaminoglycans on the mouse ZP, and terminal β-linked GlcNAc sequences are only marginally expressed on N- and O-linked glycans in this matrix [19,67].

Compelling evidence for carbohydrate recognition during murine sperm-egg binding has now been obtained in several studies. Sperm binding to murine eggs decreases by 70% following

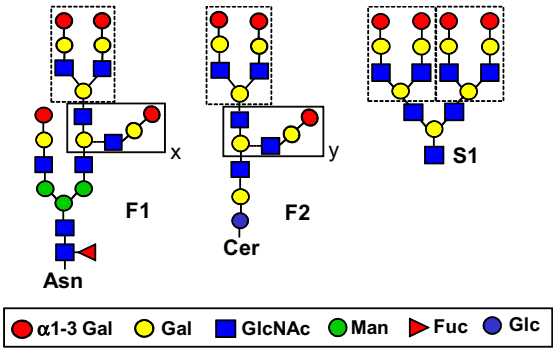

Fig. 3. Branched polylactosamine type oligosaccharides referred to in the text. Formulas for N-glycans bearing polylactosamine sequences linked to N-glycans (F1) and glycosphingolipids (F2) associated with rabbit erythrocytes are displayed. The branched sequence in the solid box is repeated *x* and *y* times, where *x* is equal to and 0–6 and *y* is equal to 0–7. The terminal heptasaccharide sequence that is shared by all of these oligosaccharides is shown in each dashed box.

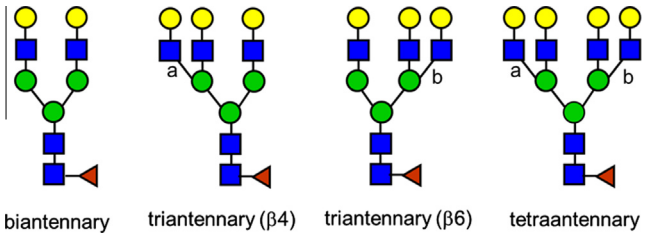

Fig. 4. Complex type N-glycans expressed in mouse and porcine ZP that act as ligands for sperm-egg binding. The terminal lacNAc (Galβ1-4GlcNAc) sequences on these N-glycans are attached via β1–2 linkages with the exception of β1–4 (A) and β1–6 linkages (B) as indicated.

exhaustive digestion with a position specific β1–4 galactosidase [67]. Similar treatment of mouse ZP with α-galactosidase does not have any effect on binding. By contrast, digestion of eggs with a sialidase that exposes underlying β1–4 linked galactose residues results in a 30–40% increase in sperm binding [67]. Simple tri- and tetrasaccharides terminated with Galβ1–4GlcNAc or Galα1–3Galβ1–4GlcNAc inhibit murine sperm-egg binding by a maximum of 74–78% at relatively high concentrations [68]. A branched polylactosamine construct terminated with either Galβ1–4GlcNAc or Galα1–3Galβ1–4GlcNAc maximally inhibits binding by 70–75% at a relatively low concentration (4 μM) (Fig. 3, S1) [69]. These findings indicate that about 75–80% of all sperm binding to murine oocytes is carbohydrate dependent, with the remaining binding due to protein–protein interactions [43]. As noted previously, results obtained in the human model indicate this same proportionality in binding via lectin-like versus protein–protein interactions, respectively [15].

Data obtained in a somatic cell adhesion system provides additional evidence supporting the concept that mouse sperm express an EBP that has affinity for glycans terminated with either Galβ1–4GlcNAc or Galα1–3Galβ1–4GlcNAc. Mouse sperm bind robustly to rabbit erythrocytes via recognition of oligosaccharides presented on their plasma membrane [70,71]. Analysis of protein- and lipid-linked oligosaccharides derived from rabbit erythrocytes reveals an abundance of extended branched polylactosamine type chains terminated with Galα1–3Galβ1–4GlcNAc at each non-reducing end (Fig. 3) [72,73]. The terminal heptasaccharide sequence on these rabbit polylactosaminoglycans inhibits murine sperm-egg binding by 70–75% when it is expressed in divalent presentation on artificial oligosaccharide construct S1 at 4 μM (Fig. 3) [11,69].

Gene deletion experiments also provide substantial support for a role for carbohydrate recognition in murine sperm-ZP binding. Global inactivation of N-acetylglucosaminyltransferase V (Mgat5) results in the generation of mutant mice that display a 40–50% loss in fecundity [74]. Conditional deletion of N-acetylglucosaminyltransferase I (Mgat1) in murine eggs results in: (i) a complete loss of complex and hybrid type N-glycans; (ii) an 81% decrease in sperm-ZP binding; and (iii) a 50% loss of in fecundity [75,76]. This combination of oligosaccharide inhibition data and the results from genetic manipulation studies indicate that tri- and tetraantennary N-glycans terminated with lacNAc (Galβ1–4GlcNAc) mediate murine sperm-egg binding, a binding specificity initially proposed in 2006 (Fig. 4) [43]. Triantennary and tetraantennary N-glycans terminated with lacNAc or polylactosamine sequences are also ligands for galectin-1 and galectin-3, two immune deviating molecules [77,78]. Again, this linkage is consistent with the SRS model.

Table 2
Proposed murine egg binding proteins.

Adhesion molecule	References
β1–4 Galactosyltransferase	[57]
56 kDa galactose binding protein (sp56)	[81]
95 kDa zona receptor kinase	[82]
Fucosyltransferase	[83]
Sulfoglycolipid Immobilizing protein	[84]
α-Mannosidase	[85]
α-Fucosidase	[86]
SED1	[87]
ZPB1 and ZBP2	[88]
Proacrosin	[89]

Dean and colleagues proposed that mouse sperm-ZP binding relies on the formation of a supramolecular complex of ZP glycoproteins rather than carbohydrate recognition [79]. However, eggs from transgenic mice that express human rather than mouse ZP3 bind murine but not human sperm [79]. Related transgenic studies were performed involving the substitution of mouse ZP2 or both mouse ZP2 and ZP3 with their respective human analogues. In both cases, murine rather than human sperm bind to humanized eggs derived from these transgenic mice [80]. As noted previously, the binding of murine sperm to eggs derived from conditional Mgat1 knockout mice decreases 81% compared to eggs obtained from wild type mice. This diminished binding was reported by Dean and colleagues [75]. In summary, the results of these genetic manipulations are completely consistent with a role for carbohydrate recognition during murine sperm-ZP binding.

Many EBPs have been proposed in the mouse model (Table 2) [57,81–89]. However, none of these adhesion molecules have been shown to mediate carbohydrate dependent binding. As noted previously, interest in the mouse model of gamete binding has declined appreciably over the past decade, making it very unlikely that any lectin-like EBPs will be identified in the foreseeable future.

4. Porcine sperm-ZP binding

Hedrick and coworkers initially isolated porcine ZP from pig ovaries in 1978 [90,91]. There are many biological and structural differences between murine and porcine ZP. Solubilized murine ZP maximally inhibits mouse sperm-egg binding by 85% at a concentration of 15 μg/ml, whereas the corresponding porcine preparation inhibits boar sperm-egg binding by only 54% at 50 μg/ml [48,92]. Purified murine ZP induces high levels of acrosomal exocytosis at relatively low concentrations (8 μg/ml), whereas porcine ZP is a much weaker activator of this reaction at a much higher concentration (125 μg/ml) [50,93,94]. These results indicate

that the spatial presentation of carbohydrate sequences could be essential in the porcine interaction.

A major experimental impediment was the inability to resolve the porcine ZP glycoprotein by gel electrophoresis, a problem that was not encountered with murine ZP glycoproteins [47]. This obstacle was finally overcome by digesting solubilized porcine ZP with endo-β-galactosidase, an enzyme that selectively depolymerizes the neutral and sulfated polylactosamine sequences associated with this matrix [95]. This enzymatic modification enables the separation of three glycoproteins designated ZP2, ZP3 and ZP4 without affecting the biological activities of these glycoproteins.

No single highly purified porcine glycoprotein blocks boar sperm-ZP binding in vitro. However, a heterocomplex of ZP3 and ZP4 exhibits this activity, but other potential binary combinations of these glycoproteins do not [92,96]. Recombinant porcine ZP3 and ZP4 synthesized in an insect baculovirus system is available, but this heterocomplex does not inhibit porcine sperm-egg binding [97]. This finding confirms that the native glycosylation of one or both of these porcine ZP glycoproteins is essential for boar sperm binding. To further test this hypothesis, Yonezawa and colleagues analyzed heterodimeric complexes of recombinant and native forms of porcine ZP3 and ZP4 to determine their effects on boar sperm binding activity [98]. They report that the heterodimer of recombinant ZP3 and native ZP4 inhibits boar sperm-egg binding, whereas the complex of native ZP3 and recombinant ZP4 does not [98]. This result confirms that the native glycans linked to ZP4 are essential for the boar sperm binding activity of the ZP3/ZP4 complex.

Unlike mouse ZP, porcine ZP could be readily isolated in substantial amounts from ovaries obtained from abattoirs. This abundance enabled more detailed analyses of the N- and O-glycans derived from this matrix compared to the mouse. Porcine ZP expresses biantennary, triantennary and tetraantennary complex N-glycans, but no high mannose or hybrid structures [23,25,99,100]. Neutral N-glycans from this matrix are unusual because 39% possess one or more antenna terminated with β-linked GlcNAc rather than lacNAc [101]. Core 1 sequences are abundant in the neutral O-glycan pool, with smaller amounts of Core 3 structures [102]. Acidic N-glycans consist of polylactosamine sequences of different length sulfated at the C-6 position of GlcNAc, making them identical to keratan sulfate sequences that are linked to proteoglycans in extracellular matrices [103]. Most of these chains are also terminated with sialic acid (α2–3 linked NeuAc/NeuGc). Sulfate groups are also linked at the C-6 position of GlcNAc in terminal H type 2 and Lewis^x sequences expressed on N-glycans [104]. Sulfated polylactosamine sequences identical to those associated with N-glycans are also abundant on O-glycans, where they are linked to core 1 structures [24,105].

The highly sulfated polylactosamine type O-glycans isolated from porcine ZP were initially implicated as the ligands that mediate boar sperm binding [106]. However, subsequent studies did not support this binding specificity. Nakano and colleagues obtained evidence in several reports that boar sperm bind to tri- and tetraantennary N-glycans capped with lacNAc (Galβ1–4GlcNAc) [100,107–109]. As noted earlier, these biologically active N-glycans are linked specifically to ZP4 [98]. The precise glycopeptide domain within ZP4 that mediates binding has been defined [110]. These findings indicate that both mouse and porcine sperm bind preferentially to tri- and tetraantennary N-glycans terminated with lacNAc (Fig. 4). This potential overlap was further tested by incubating mouse sperm with porcine eggs, where they bind with the same avidity and in the same number as boar sperm [111]. Boar sperm also bind to rabbit erythrocytes with the same high affinity as murine sperm do [111]. However, unlike murine sperm, boar sperm rapidly undergo acrosomal exocytosis soon after binding to this somatic cell type. Since there are no proteins on rabbit erythrocytes with porcine ZP domains, this finding indicates that carbohydrate recognition could play a role in the induction of acrosomal exocytosis in the pig.

Table 3
Proposed porcine egg binding proteins.

Adhesion molecule	References
Fucose binding protein	[112]
Sperm protein 38	[113]
Zonadhesin	[114]
APz	[115]
Proacrosin	[116]
Spermadhesins (AWN, AQN-3)	[117]
pAIF	[118]
P47	[119]
Peroxiredoxin	[120]
Fertilin-β	[120]

There are also many different EBPs that have been proposed for the pig (Table 3) [112–120]. Porcine ZP binds many different sperm membrane proteins, among them AQN-3, P47 (SED-1), fertilin-β and peroxiredoxin-5 [118,120,121]. Hardy and coworkers identified another adhesion molecule designated zonadhesin that also binds to porcine ZP [114]. However, only the spermadhesins express carbohydrate binding domains [122]. AQN-3 binds to both tri- and tetraantennary N-glycans terminated with lacNAc, consistent with the proposed role of these carbohydrate sequences in mediating sperm binding in this species [123]. In summary, the most potent case for a physiological lectin-like EBP (AQN-3) has been obtained in the pig model.

5. Historical perspective and future directions for research

Minkin and Rock reported their observations of human sperm-egg binding, fertilization and early embryonic development in vitro seventy years ago, generating a media sensation [124]. Scientific interest in mammalian sperm-egg binding interactions was substantially increased by the successful development of traditional human IVF in 1978 [125]. In the 1980s, human IVF became a routine medical procedure practiced in many clinics. Careful analysis of human IVF cases revealed deficient sperm-ZP binding as a major cause of failed fertilization [126–128]. This clinical problem provided a strong impetus for an effort to define the molecular basis of human sperm-ZP binding. The hope was that such studies could enable the identification of men at risk for such defects and eventually lead to clinical solutions to restore male fertility.

Biomedical research efforts to understand human sperm-ZP binding largely bifurcated along two experimental paths. Basic scientists proposed that this interaction should be investigated in animal models like the mouse and pig first because of far greater access to material and the ability to perform experiments that would be unethical in humans and other closely related sentient hominids. In addition, methods for the isolation of substantial amounts of mouse and porcine ZP were developed in the same year that the first successful human IVF was achieved [90,129]. The tacit assumption at that time was that any findings made in the mouse model would be fully applicable to the human model [40,130,131]. Ten experimental models for the murine sperm-ZP interaction were eventually proposed (Table 2) [11,43,46,132].

By contrast, several clinically oriented research groups chose to experiment with the limited number of human eggs that failed fertilization and those collected from cadavers [133–135]. Such efforts were greatly aided by the development of the human HZA, an internally controlled bioassay that could be reliably used to quantitate human sperm-ZP binding in vitro [136]. The technique greatly expanded the number of experiments that could be performed with the limited number of available human eggs. When executed under

carefully controlled conditions, binding activity in the human HZA was found to be an excellent predictor of success in traditional IVF [137,138]. Unfortunately, the HZA could never be routinely employed in the clinical setting due to the inadequate number of available human eggs and the high technical costs associated with the cellular manipulations required for its execution.

Human gamete binding remained a topic of major clinical interest throughout the 1980s. However, the development of the intracytoplasmic sperm injection (ICSI) method in 1992 circumvented all the sperm binding events necessary for fertilization, enabling successful IVF outcomes regardless of the ability of sperm to bind and penetrate the ZP or fuse with the oocyte [139,140]. For many years, there were concerns that ICSI/IVF would lead to higher rates of birth defects than traditional IVF. However, careful analysis of the results has confirmed that ICSI-IVF and traditional procedures lead to an equivalent increased risk of birth defects [141].

The development of ICSI substantially diminished clinical interest in defining the molecular basis of human sperm-ZP binding. There are several other factors that have further decreased enthusiasm for such investigations. As noted previously, studies performed in the human HZA have confirmed that about 80% of human sperm binding involves carbohydrate recognition, with the remainder relying on protein–protein interactions [15]. These results indicate that two or more separate EBPs with different binding specificities likely exist. Nonetheless, the possibility remains that there is a single protein or heterodimeric complex that bears both lectin-like and protein binding domains that interact with human ZP glycoproteins. Therefore the development of potent contraceptive agents that target initial human sperm-ZP binding could require at least two and possibly more targets. In addition, the exact threshold of human sperm-ZP binding that must be eliminated to block fertilization is currently unknown, but could be quite substantial [15,43].

Another complicating factor is the observation that human ZP glycoproteins are densely coated with SLEX. These terminal sequences likely have a major influence on the biochemical and physiological properties of human ZP glycoproteins, suggesting that recombinant human ZP glycoproteins lacking such sequences are unlikely to be physiologically relevant. Thus native human ZP or recombinant ZP glycoproteins terminated with SLEX sequences are likely required to identify and isolate physiologically relevant EBPs. The number of available human eggs is highly limited, and recombinant ZP glycoproteins bearing naturally occurring human ZP glycans have not yet been reported.

Despite these numerous difficulties, research focused on defining the molecular basis of human sperm-ZP binding should remain a high priority. The world's population is currently 7.22 billion, and is increasing annually at a rate of 1.14% (World POPClock Projection, 2014). This expanding population will only add to CO_2 emissions that will accelerate the rate of global climate change. This coupling suggests that major efforts should be made to decrease CO_2 emissions and promote zero population growth. Unfortunately, due to the intense lobbying efforts by the fossil fuel industry and the indifference of some politicians in developing countries to address the issue of overpopulation, it is now rather unlikely that severe damage to the global environment can be avoided. A safe, easy, reliable, inexpensive, and reversible method of birth control is sorely needed to eliminate population growth and reduce environmental stress.

There could also be a direct clinical benefit of such research. The human HZA is a useful sperm function test that is highly predictive of male fertility and success in the traditional IVF setting [126,136–138]. As noted previously, the human HZA will forever remain an experimental procedure because of several logistical and cost constraints. The development of beads coated with human ZP glycoproteins that bind sperm and induce acrosomal exocytosis in these gametes would represent a useful sperm function test to screen patients in the andrology clinic.

In summary, there has been a considerable research effort to understand initial mammalian sperm-ZP binding since 1978, but biomedical interest in this topic has diminished substantially since the introduction of the ICSI procedure for human fertilization. The complexity of this binding interaction has further decreased interest in developing useful contraceptives that target initial human sperm-ZP binding. The effort to understand this earliest stage of mammalian fertilization is now effectively stalled. Our understanding of initial sperm-ZP binding will remain incomplete until this information is obtained. The arguments offered here should stimulate more support for this area of research in the future, but whether they will or not is uncertain.

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