Interaction of sperm with the zona pellucida during fertilization

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In order to achieve fertilization sperm cells, first need to successfully interact with the zona pellucida. To this end, the sperm surface is extensively remodeled during capacitation and the resulting sperm cells also possess hyperactivated motility. Together, this serves to mediate optimal recognition of the zona pellucida in the oviduct or after in vitro fertilization incubations (primary zona pellucida binding). When the sperm cell attaches to the zona pellucida, it will be triggered to undergo the acrosome reaction which allows the hyperactivated motile sperm cell to drill through the zona pellucida (secondary zona pellucida binding coinciding with sequential local zona pellucida digestion and rebinding). After successful zona penetration, some sperm cells may enter the perivitelline space. This delaying strategy of the oocyte allows only one sperm cell at a given time to bind and fuse with the oocyte (fertilization) and thus minimizes the risk of polyspermy. The fertilization fusion between the oocyte and the first sperm cell is immediately followed by a polyspermic fertilization block, in which the content of the oocyte's cortical granules is released into the perivitelline space. The cortical reaction blocks further sperm-oocyte fusion either by sticking at the oolemma or by the induction of a biochemical reaction of the zona pellucida (zona pellucida hardening). The cortical reaction thus blocks sperm-zona pellucida binding and/or sperm-zona pellucida penetration. This review summarizes the current understanding of sperm-zona pellucida interactions in relation to mammalian fertilization. The lack of knowledge about sperm-zona pellucida binding in ruminants will be critically discussed.

The zona pellucida

The mouse zona pellucida

Most of our current understanding of the zona pellucida is based on data obtained from the mouse species (for a recent review see Wassarman and Litscher 2009). Like in other mammals the extracellular matrix of the oocyte has three peculiar properties: (1) It is quite thick for an extracellular matrix (in rodents around 7 μm); (2) It is not attached directly to the oocyte but to the cumulus cells surrounding the periphery of the oocyte, leaving a cell free space commonly termed perivitelline space between the glycocalyx and the oolemma; and (3) Isolation and solubilization of the zona pellucida revealed that the mouse zona consists of only three zona proteins (ZP1, ZP2, ZP3), all of which are heavily and differentially glycosylated (Wassarman 1988).

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The mouse zona pellucida is formed by linear filaments of ZP 2 and 3 that are cross linked by ZP1. Zona protein treatments with chymotrypsin, or with reducing agents, altered the functional integrity of ZP1 and as a result filaments of ZP2/ZP3 could be isolated from mouse ZP (Greve and Wassarman 1985). Such filaments are actively being secreted by the oocyte forming linear arrangements attached to the oolemma of developing oocytes (Wassarman and Albertini 1994). At a later phase, the characteristic thick (Wassarman 2008) zona pellucida is formed and between this structure and the oolemma a cavity (the perivitelline space) is created (Wassarman and Albertini 1994). Recent studies on structural features of ZP proteins revealed that, beyond the secretion domain present on the translated preZPs, the secreted ZPs also contain a ZP domain containing a conserved sequence (ZP-N domain at the N terminal end) that is involved in forming polymers that are shared with other extracellular matrix proteins (Jovine et al., 2006). How this ZP-N domain (with PLAC-1 homology Jovine et al., 2007) is involved in ZP polymer formation has recently been elucidated with ultrastructural techniques (Monné et al., 2008).

Solubilization of the zona pellucida (by heat treatment at low pH) and functional studies with the three individual purified zona proteins revealed that ZP3 is the receptor for intact sperm and interacts with sperm surface proteins present in the apical head region (*primary zona pellucida binding*) and that ZP3 is also responsible for induction of the acrosome reaction (Litscher and Wassarman 1996; Buffone et al., 2009a). The c-terminal fragment (ZP-C domain) of ZP3 is also thought to be involved in sperm binding (Wassarman 2008). Specific glycan structures on ZP3 appear to be involved in the zona pellucida-induced acrosome reaction as this response can be mimicked in vitro by adding Lewis X to sperm or by supplementing albumin with covalently linked carbohydrates (neoglycoproteins) (Bendahmane and Tulsiani 2003, Loeser and Tulsiani 1999, Hanna et al., 2004; Kerr et al., 2004, Williams et al., 2007). However, the involvement of *N*- and *O*-glycans on mouse ZP3 for primary sperm binding and fertilization has recently been challenged using transgenic mice that expressed a mutated ZP3 that lacked glycosylation sites. These transgenic mice had normal fertilization rates and normal litter sizes (Gahlay al., 2010).

ZP2 is the receptor that is involved in *secondary zona pellucida binding* as it interacts only with acrosome reacted cells as those cells have exposed acrosomal proteins involved in zona pellucida binding and penetration (Bleil et al., 1988; Howes et al., 2001). While ZP2 is not only involved in the secondary zona pellucida binding event, it also is believed to become cleaved by acrosomal enzymes allowing sperm drilling through the zona pellucida (Moreno et al., 1999) although not much is known about how acrosome proteins mediate zona digestion. For instance it is not known whether the ZP2 processing by acrosomal enzymes is comparable to the formation of ZP2f after fertilization (see section on zona hardening).

Following successful penetration through the zona pellucida, only one sperm will fertilize the oocyte and this one event is immediately followed by the cortical reaction which will initiate *zona pellucida hardening*. Zona pellucida hardening essentially blocks penetrating sperm from proceeding to the perivitelline space. In the mouse, both ZP3 and ZP2 have been shown to be modified into truncated forms: ZP3f and ZP2f, respectively. The ZP3f in its solubilized form did not possess affinity for sperm cells nor the capacity to induce the acrosome reaction (Bleil and Wassarman 1983). The formation of ZP2f is less understood but has been implicated to be functional for zona pellucida hardening (Schroeder et al., 1990; Kalab et al., 1991). Recently, the folding properties of the ZP-N domains of all three ZP proteins has shed new light on the possibility that the cortical reaction mediated modifications to ZP proteins includes re-folding of these domains and thus may be functional in zona hardening (Jovine et al., 2007; Monné et al., 2008). In fact, the folding of ZP2 has been shown to also be essential for primary sperm-zona binding (Gahlay et al., 2010)

Zona pellucida composition in other mammals (especially bovine and porcine)

In pigs, cattle, and other mammalian species the terminology used for ZP proteins varies in the literature despite the fact that the ZP proteins are functionally homologous to the murine system as described by Wassarman (see previous section). Classically, mouse ZP (mZP) proteins have been functionally defined as mZP1 (the cross-linker of linear mZP2-mZP3 polymers), mZP2 (involved in secondary sperm zona binding), and mZP3 (involved in primary sperm-zona binding). In recent literature for human and porcine species, new names for these protein have been proposed based on the length of the cDNA matching each ZP transcript (ZP-A for mZP2; ZP-B for mZP1 and ZPC for mZP3). However, new studies have shown that ZP-B (Conner et al., 2005, Lefièvre et al., 2004; van Gestel et al., 2007) is a fourth ZP protein in primates, porcine, ruminants, and rabbit with sperm binding proteins similar to ZP3 (this protein is absent in murine zonae pellucidae). In recent human literature ZPB is called ZP4 (Gupta et al., 2009, Ganguly et al., 2010) and a pseudogene for this has been found in rodents (Gupta et al., 2009). In cattle, pigs, and humans, ZP3 and ZP4 are involved in sperm binding and induction of the acrosome reaction (Gupta et al., 2009; Chiu et al., 2008a). ZP2 is involved in secondary sperm binding (i.e. after the acrosome reaction; Chiu et al., 2008b) and indirect observations on human material with antibodies raised specifically against ZP1 (this protein is not observed in porcine and bovine zona pellucida) blocked sperm binding but this could be due to steric hindrance of the antibody used towards ZP3/4 binding (Ganguly et al., 2010). In bovine literature, the analogs for human ZP-3 and ZP4 (or ZPB) were called bZP3α and bZP3ß, respectively (Topper et al., 1997) and the 3 forms of the zona protein analogous to human ZP2 were called bZP1, bZP2 and bZP4 (and similar nomenclature was used for porcine zona protein pZPs see Figure 1). As depicted for porcine zona pellucida (Figure 1), the bZP2 has the same polypeptide backbone but lacks a 25 kDa glycoprotein (bZP4) portion of bZP1. The zona pellucida of mouse is composed of ZP1, 2 and 3; however, other rodents such as the hamster also contain ZP4. The human zona pellucida is composed of all four zona proteins (ZP1-4) while bovine and porcine have ZP2, 3 and 4 as proteins, but do not contain ZP1 (Kanai et al., 2008). The older nomenclature should not be used anymore to avoid confusion. For a review of zona pellucida protein composition, nomenclature, and the phylogenetic relationship and diversity of these proteins in vertebrates see Goudet et al., 2008 and Izquierdo-Rico et al., 2009. In all mammalian species mentioned so far, the expressed ZP proteins form a 7 μm thick three dimensional network. In the porcine and bovine species no ultra-structural studies have been conducted to determine whether chymotrypsin or reducing treatments caused dissociation of the three dimensional zona matrix into filaments (as observed for rodents, Greve and Wassarman 1985).

Likely, ZP3 and ZP4 form such filaments by disulfide bridges (Kanai et al. 2008). In vitro, ZP3/ZP4 heteromers have affinity for sperm and are able to induce the acrosome reaction. Glycosylation of these proteins is supposed to be important in both sperm binding as well as in the induction of the acrosome reaction (Chakravarty et al., 2008, Yonezawa et al., 2007). In bovine, α-mannosyl residues on the *N*-linked carbohydrate chains of the zona pellucida glycoproteins have been reported to be essential for sperm binding (Amari et al., 2001). In humans, recombinant ZP4 was sufficient for sperm binding and could induce the acrosome reaction (Chiu et al., 2008a). In fact, the signaling pathways to evoke the acrosome reaction were not identical to that of ZP3. The ZP3 and ZP4 induced acrosome reactions were especially *N*-linked glycosylation dependent and in the sperm they were protein kinase-C, protein tyrosine kinase, T-type Ca^{2+} channels, and extracellular Ca^{2+} dependent. G-proteins also participated in human ZP3- but not in ZP4-induced acrosome reaction (Chiu et al., 2008a). On the other hand, protein kinase-A and L-type Ca^{2+} channels took part only in human ZP4-induced acrosome reaction (Chiu et al., 2008a). Moreover, recombinant bovine or porcine ZP3/ZP4 heterocom

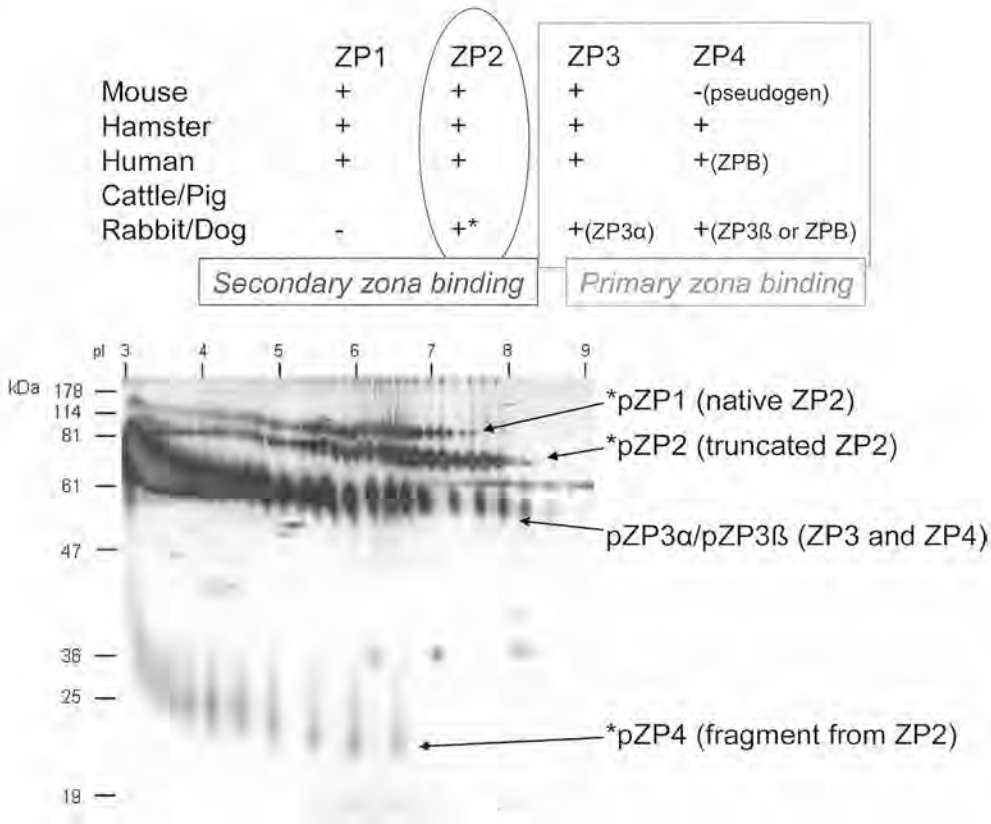

Fig. 1. Upper panel: Comparison of zona pellucida protein composition of the species mentioned in the text. ZP2 is involved in secondary zona binding, whereas, ZP3/ZP4 are involved in primary zona binding and induction of the acrosome reaction. Note the similarity of bovine and porcine zona pellucida protein composition which is different from mouse, hamster, and human in that it does not contain ZP1. **Lower panel: Two dimensional gel electrophoretic separation of solubilized porcine zona proteins.** The multi-glycosylation especially with negative charged carbohydrate chains cause the remarkable acidic shift and the very characteristic so called glycosylation trains of solubilized ZP after 2D IEF/SDS-PAGE of porcine ZP. The porcine nomenclature is indicated with p as a prefix. Between brackets the new nomenclature for ZPB (now named ZP4) is provided. The pZP2 isoform of ZP2 lacks approx. 25 kDa (pZP4) while pZP1 still includes this structure but all three forms are in fact originating from ZP2. The same components as named here were found in bovine ZP with different MW and pI as well as different relative compositions and the same terminology has been used with b as prefix (Topper et al., 1997). Porcine zona proteins are more differentially glycosylated and more charged than bovine or human zona proteins (Hedrick and Wardrip 1986; Koyama et al., 1991). This 2D gel was originally published by van Gestel et al., 2007, and is used with modifications to illustrate the complex carbohydrate nature of the zona pellucida and serves for the reader to appreciate and identify the different zona proteins and isoforms (by permission of Oxford University Press).

plexes expressed in Sf9 cells have an affinity for sperm and induce the acrosome reaction to sperm cells that have bound to these heterocomplexes (Kanai et al., 2007). The pZP4/bZP3 heteromers as well as bZP4/pZP3 heteromers retained sperm binding and acrosome reaction

induction properties, which indicates that primary zona binding is conserved between these two species (Kanai et al. 2007) and appears to be similar to the human situation (Chiu et al., 2008b). In addition, a decapeptide of bovine ZP3 can induce a G-protein activation of the acrosome reaction in bovine sperm (Hinsch et al., 2005) showing that specific structures within the zona pellucida are involved in this process. Despite the above mentioned species similarities of synthetic zona proteins in sperm-zona binding and acrosome reaction, it has been shown that differences in *N*-glycosylation of ZP3/ZP4 are responsible for causing bovine and porcine species specific sperm-zona interactions (Yonezawa et al., 2007; Yonezawa et al., 2005). The above study describes the affinity of individual or heteromeric ZP proteins but does not cover all aspects of the zona's affinity for sperm as has been demonstrated by Yurewicz et al., 1998. Based on his observations he concluded that the native ZP texture rather than solubilized and isolated individual zona proteins or ZP3/ZP4 polymers had full affinity for sperm membrane vesicles (Yurewicz et al., 1998). Remarkably, both equine and porcine sperm can bind to the native bovine zona pellucida. Porcine sperm can also induce the acrosome reaction after such binding and the sperm cell is able to penetrate this bovine zona pellucida. In contrast this is not observed for the bound equine sperm to bovine zona pellucida (Sinowatz et al., 2003). A similar phenomenon is described between interspecies sperm zona binding and zona penetration between equine and porcine zona pellucida (Mugnier et al., 2009) and between donkey sperm and bovine zona pellucida (Taberner et al., 2010). These observations challenge the species specificity of both primary and secondary sperm-zona interactions.

In bovine and porcine species, secondary sperm binding needs ZP2 and blocking this interaction prevents fertilization (Hasegawa et al., 2000) and appears to be conserved with human ZP2 (Hinsch et al., 2003). Prior to fertilization a proportion of full sized ZP2 (originally named bZP1 or pZP1) is partially cleaved into a truncated ZP2 (originally named pZP2 or bZP2) and a 25 kDa fragment (originally named pZP4 or bZP4) (Hasegawa et al., 1994; figure 1). The importance of pre-fertilization cleavage of full ZP2 into a truncated and a 25 kDa form for sperm-zona interactions is not yet established. Most likely in porcine and bovine, ZP2 interacts with ZP3 and ZP4 in a species specific manner and is different from mouse, since expressed recombinant porcine ZP2 in transgenic mice does not result in incorporated pZP2 in the resulting zona pellucida (Hasegawa et al., 2006). In contrast to the carbohydrate dependency of bovine primary zona pellucida binding (*N*-acetyllactosamine residues are important), the secondary zona binding to ZP2 was not diminished after ß-galactosidase treatments (Ikeda et al., 2002). Interestingly in humans, acrosome reacted sperm do not bind to the zona pellucida of human oocytes which could imply that primary zona binding involves molecules that are required for establishing secondary zona binding proteins (Liu et al., 2006).

Zona hardening

After primary and secondary sperm zona interactions, sperm will enter the perivitelline space and may interact and fuse with the oolemma and thus fertilize the egg. Immediately after the fertilization fusion, soluble sperm factors (like phospholipase C ξ ; Dale et al., 2010) induce intracellular Ca^{2+} events and oolemma depolarization and this is a permissive step to induce the cortical reaction. The released cortical content will affect the structure of the zona pellucida (zona hardening) preventing sperm from binding to or penetrating the zona pellucida. In mouse, it has been shown that the cortical reaction causes truncations of ZP2 and ZP3 without affecting the molecular weight of ZP1 (Wassarman and Albertini 1994). Recently, evidence has been gathered with transgenic mice expressing mutated ZP2 that lacks the cleavage site so that ZP2 cannot be cleaved into ZP2f during the cortical reaction. The removal of sperm

associated to the zona pellucida (normally a result of the cortical reaction) was not detected at the transgenic zona pellucida of two cell stage embryos (Gahlay et al., 2010).

In pigs and cattle, ZP2 is cleaved (Hatanaka et al., 1992, Noguchi et al., 1994) including a change in the disulfide bonds of this zona protein (Iwamoto et al., 1999). In addition, proteases (Hoodbhoy and Talbot 1994) have been found to be responsible for and structural rearrangements in the zona pellucida after fertilization (Funahashi et al., 2000, 2001, Nara et al., 2006). The bovine cortical reaction causes a removal of sialic acid from the carbohydrate moieties of the zona pellucida which causes a reduction in sperm-zona binding and penetration (Velásquez et al., 2007). This effect can be mimicked by adding blocking lectins for sialic acid residues or by adding *N*-acetyl-D-glucosamine (Sakaguchi et al., 2009). Supplementation of *N*-acetyl-D-glucosamine also resulted in increased acrosome reaction induction, increased secondary sperm zona binding and increased polyspermic fertilization. Similar effects were reported for cattle by using fetuin (Landim-Alvarenga et al., 2002). In porcine, the ß-galactosyl residues of the neutral *N*-linked carbohydrate chains of the zona proteins appear to be important for sperm-zona interactions (Yonezawa et al., 2005), indicating that the *N*-glycosylation of the zona pellucida is responsible for species differences between the pig and the cow (Yonezawa et al., 2007). In bovine oocytes, the efficiency of zona hardening and the prevention of polyspermic fertilization are related to the degree of maturation of the oocyte (Santos et al., 2008). In summary, this section of the review describes changes that are supposed to block primary zona binding, the induction of the acrosome reaction, secondary zona binding and sperm penetration. In humans, a fertilized oocyte with a hardened zona pellucida still has the capacity to bind sperm and to induce a voltage-dependent calcium influx along with an acrosome reaction but sperm can not penetrate the hardened zona pellucida (Patrat et al., 2006). Experiments of porcine and bovine fertilized eggs are missing in this regard.

Pre-fertilization zona modifications by oviductal proteins

It is noteworthy that hardened zona ghosts (isolated and purified remnants of ruptured zonae pellucidae) can be used as a binding control when studying sperm-zona interactions with zona ghosts obtained from unfertilized oocytes. The hardened zona ghosts can also be useful to study imperfect polyspermic fertilization blocks such as observed in IVF treatments for pigs. In this light it is interesting that prefertilization hardening of the zona pellucida reduces the risk of polyspermic fertilization in pigs and cows probably by reducing the amount of sperm that bind to the zona pellucida and delaying the speed of zona drilling which in turn will provide the just fertilized oocyte with more time to fulfill its functional cortical reaction (Coy et al., 2008a, Canovas et al., 2009). It is possible that the partial precleavage of ZP2 in porcine and bovine zona pellucida may be relevant for limiting polyspermic fertilization in a similar way (see previous section). Recently it has been acknowledged that the zona pellucida of the ovulated oocyte may be subjected to changes that influence sperm-zona interactions by secretions of the oviduct (Coy and Avilés 2009) but is not clear whether the zona properties are affected or whether the sperm cell functionality is affected (Coy et al., 2010; for review see Killian 2004). Treatments of bovine zona pellucida with antibodies directed against specific oviductal fluid proteins (either osteopontin or lipocalin type prostaglandin D synthetase) inhibited sperm binding and fertilization suggesting that such oviductal proteins at least also interact with the zona pellucida (Gonçalves et al., 2008). Furthermore, the oviduct specific glycoprotein and heparin have been shown to be responsible for post-ovulatory and pre-fertilization modification of the bovine zona pellucida that affect sperm-zona binding and appear to be involved in polyspermy regulation (Coy et al., 2008b). Recently, oviduct-specific glycoprotein has been

found as part of the mouse zona pellucida in which it acts as a ZP3 independent sperm adhesion ligand (Lyng and Shur 2009; for a recent review see Avilés et al., 2010); whether or not bovine oviduct specific binding protein works the same remains to be investigated. Similarly, another oviductal protein (also detected in the bovine oviduct) named osteopontin has been reported to reduce polyspermy in porcine (Hao et al., 2006). In addition, another oviduct component is added to the periphery of the ovulated mouse zona pellucida and provides the zona pellucida with additional sperm affinity (independent from sperm ß 1,4-galactosyltransferase I activity; Rodeheffer and Shur 2004; see section on primary sperm binding to zona).

As mentioned above, the zona pellucida proteins (and their differential glycosylation) are a matter of high evolutionary genetic drift (the same is valid for zona binding proteins at the sperm surface or in the sperm acrosome). Therefore, sperm-zona binding can be considered as species specific and variations in this interaction could form the basis of mammalian speciation. Monné et al. 2008 have resolved a specific ZP-N fold structure with implications for overall zona pellucida architecture, the post-fertilization block to polyspermy and speciation. In contrast, the fertilization fusion appears to be much less species specific. For instance, in the hamster oocyte assay in which a zona denuded hamster oocyte is used to assess the fertilization fusion capability of human sperm (Barros et al., 1979). The zona pellucida of mammalian oocytes has to a large extent been studied in only a few species namely domestic animals such as the ewe, cow and sow (from these species ovaries can be obtained relatively easily and from a large amount of animals at a slaughter line) and from laboratory mammals such as the rat, mouse and Guinea-pig. Studies on large quantities of oocytes are required for the type of biochemical studies but provide only a raw view. The ultimate changes of the zona pellucida at the last stages of oocyte maturation (peri-ovulation) and before fertilization in the oviduct are not studied in detail but may well be crucial for obtaining optimal fertilization efficiency both in enhancing the fertilization efficiency (in vitro, this is in the range of 30-60 %; in vivo >85%), but also to prevent polyspermic fertilization (which is relatively frequent in pig IVF) (Hao et al., 2006). Therefore, the use of oviduct secretions in future assisted reproductive technologies is foreseen (Avilés et al., 2010).

The sperm cell

In vitro capacitation:

Before sperm cells enter the isthmus and move up to the ampulla where they may meet the cumulus oocyte complex, they face a series of surface remodeling steps that enables them to interact with the zona pellucida and to induce the acrosome reaction (for review see Tsai and Gadella 2009). Most knowledge on sperm proteins interacting with the zona pellucida has been obtained under in vitro fertilization conditions. Basically, the sperm cell has zona binding properties before ejaculation as has been shown for aspirated sperm from the cauda epididymis (see Tsai and Gadella 2009 for review). In fact, several proteins secreted by the epididymis become adsorbed to the sperm surface (Zanich et al., 2003) and some of them remain hydrophobic interacting (mostly GPI anchored proteins) with sperm after capacitation and have a function in zona binding (Morin et al., 2010; Busso et al., 2007; van Gestel et al., 2007; Shur et al., 2006; Montfort et al., 2002). Aside from these additions, also sperm surface proteins (mostly trans membrane proteins) originate from the testis (Busso et al., 2007, van Gestel et al., 2007).

The above mentioned zona binding proteins reside at the sperm surface but become coated by seminal plasma factors during ejaculation. In the boar this coating serves to modify and sta-

bilize the sperm surface and thus to extend the sperm cell's resistance to stress (see for review Muiño-Blanco et al., 2008), which is probably a physiological requirement for sperm to survive the passage through the female reproductive tract. The seminal coating of glycoproteins also prevents premature activation of sperm cells and therefore can be considered as decapacitation factors (Vadnais and Roberts 2010, Caballero et al., 2005). Recently, decapacitation factors in the mouse were identified by proteomics technology and included plasma membrane fatty acid binding protein, CRISP-1, phosphatidylethonalamine binding protein 1 (Ensslin et al., 1995) and the yet functionally unknown decapacitation factor 10. All four secreted proteins inhibit both zona binding and zona induced acrosome reaction (Nixon et al., 2006).

Remarkably, although bovine seminal plasma proteins also stabilize sperm cells (Manjunath et al., 2007), the knowledge regarding decapacitation factors is very poor for ruminants, probably because inhibiting effects of seminal plasma proteins have not appropriately been tested yet. Instead, a family of gelatin binding proteins are the major secretory proteins recovered from bovine seminal plasma (BSP-A1, BSP-A2, BSP-A3 and BSP-30) and appear to function as pro-capacitation factors under the proper fertilization conditions either in presence of lipoproteins or in presence of heparin (for review see Manjunath et al., 2007). These proteins also form major components of seminal plasma in ram (Bergeron et al., 2005), and in buck (Villemure et al., 2003).

During the processing of sperm for IVF, the sperm are first washed through a discontinuous density gradient (made from isotonic cushions of Percoll, Ficoll or related materials), which for non-ruminant mammals at least serves to remove decapacitation factors rendering such cells responsive to in vitro fertilization incubations. The washed cells are exposed to higher temperature (39 °C instead of the epididymal 35 °C) and higher bicarbonate concentrations (>15 mM compared to <5 mM) in the ejaculate and lipid modifying proteins (mostly fatty acid free bovine serum albumin). When incubated for a couple of hours, a subpopulation of sperm cells becomes capacitated (capacitation in vitro) and these types of changes appear to mimic what is happening with sperm cells in the oviduct (for reviews Gadella and Visconti 2006; Tsai and Gadella 2009). This procedure is standard for mammalian IVF and in ruminants the addition of glycosaminoglycans, such as heparin, is required for obtaining full capacitation (Parrish et al., 1986). The IVF procedure is believed to resemble what is happening in the oviduct which is reflected in the name used for the IVF incubation medium called synthetic oviduct fluid (SOF medium). The IVF incubations do not only cause sperm surface stripping of seminal plasma components but include delicate surface alterations (Tsai and Gadella 2009; Gadella et al., 2008) and thus induces a remodeling of lipid membrane protein topology especially in the sperm head. Typically, at the apical ridge area of the sperm surface rearrangements include the aggregation of lipid ordered domains and the build up of a zona binding complex (for reviews see Boerke et al., 2008; Tanphaichitr et al., 2007). Although specific raft forming lipids allow formation and aggregation of rafts, they probably do not play a direct role in zona binding, but more likely form the template for allowing the formation of the zona binding protein complex (for discussion see Nixon et al., 2007). The apical sperm surface specific aggregations of lipid rafts have been observed in capacitating mouse, porcine and bovine sperm (Thaler et al, 2006; van Gestel 2005, 2007, Selvaraj et al., 2007). Our group has shown that capacitation resulted in the exposure and assembly of surface molecules into a high zona affinity complex of proteins in the aggregating lipid ordered apical head surface of porcine sperm (van Gestel et al., 2007). Recently, we also showed that this coincided with the stable docking of the acrosome at the same sperm surface area (Tsai et al., 2007, 2010). Probably this serves as a preparative step of sperm to immediately induce the acrosome reaction after zona recognition.

Primary zona binding

Collectively, the above mentioned in vitro capacitation changes at the sperm head surface are required to establish proper primary zona binding and the immediate induction of the acrosome reaction. Both are induced by ZP3/ZP4 (see zona pellucida section) and are exclusively executed at the apical sperm head surface (Tsai and Gadella 2009). A number of different ways have been described in order to study primary sperm-zona binding (for an overview, see van Gestel et al., 2007). One of the first concerns for a biochemical approach is that once a sperm has attached to the zona pellucida it will undergo the zona-induced acrosome reaction which is immediately followed by the initiation of massive secondary sperm-binding by intra-acrosomal glycoproteins and zona drilling. In order to avoid this, assays have been developed in which primary zona binding is blocked by preincubation of the zona pellucida with (i) the (labeled) sperm protein being studied, peptides derived from the protein of interest, preincubating the sperm with antibodies directed against the protein of study or with preincubating sperm with antibody fragments thereof. Unfortunately, all such approaches can only deliver indirect evidence for the involvement of sperm surface proteins, simply because aspecific capping of the epitopes required for zona recognition may explain inhibited sperm-zona binding. In fact, both the zona pellucida and the apical sperm plasma membrane should be isolated from the sperm and the oocyte, respectively. This allows direct biochemical characterization of primary zona binding proteins from the sperm surface. The two interacting structures can be isolated in large amounts. With this respect, porcine and bovine can be considered as model species since zona material can be obtained directly from the slaughter line in large amounts and both species produce cell rich ejaculates with >85 % normal matured cells (Gadella 2009). These approaches are described Below.

Isolation of the zona pellucida and solubilization of such proteins and immobilization to column material can be followed by affinity chromatography using sperm membrane proteins (Lea et al., 2001; Ensslin et al., 1998). One should be careful to collect ovaries, isolate oocytes from it and purify separated zona pellucida ghosts from that material, without the occurrence of the cortical reaction. The cortical reaction would alter the zona texture and modify the zona proteins (see section about zona hardening). The cortical reaction is prevented routinely by collecting and processing the material in ice cold buffers containing high amounts of EDTA (Dunbar et al., 1980; Topper et al., 1997). Rather than solubilizing the zona pellucida it has been recognized that the complex differential protein glycosylation (see for instance Fig. 1) and the native quarternary structure are essential for accurate primary zona recognition by sperm (Yurewicz et al., 1998; Nakano and Yonezawa 2001) and therefore the native zona isolated from oocytes is preferably used as a template for sperm membrane proteins.

From the sperm cell, only the surface should be used in order to prevent the identification of secondary zona binding proteins from the acrosome (see next section). Two reviews describe pro's and con's for isolating sperm membranes (Gadella 2009; Brewis and Gadella 2010). Basically the method of choice is to perform nitrogen cavitation and differential centrifugation to isolate only the apical sperm plasma membrane; whereas, the acrosome remains intact and distal sperm head membranes as well as plasma membrane of the tail and mid-piece remain associated to the remaining sperm (Flesch et al., 1998). Much less specific results are observed when hypo-osmotic mechanic disruption of sperm is performed as has been done in mammals that produce lower amount of sperm (aspiration from epididymis such as is required for murine species or <30 % normal sperm in case of many primates, for discussion see Gadella 2009).

When zona ghosts and apical sperm membranes are allowed to bind, a number of proteins could be identified as directly involved in primary sperm zona binding proteins using specific membrane proteomics techniques (see van Gestel et al., 2007, Gadella 2009, Brewis and Gadella

2010) and a number of identified proteins were reported in the literature from more indirect methods or with biochemically less specific material. A new approach to identify bovine sperm membrane proteins that interact with receptors on the oocyte and its vestments has not been very successful as membrane proteins are notoriously difficult to solubilize and therefore per definition not so suitable for 2D gel ectrophoretic separation. Consequently, predominantly soluble proteins were identified (Pate et al., 2008). Modern off-gel techniques in combination with the above mentioned isolation of interacting structures will provide functional insights in primary zona binding (Brewis and Gadella unpublished). Currently, a complete compilation of sperm surface proteins involved in primary zona binding is not available for ruminant mammals.

Two transmembrane proteins involved in primary zona binding that originate from testicular germ cells are fertilin ß (previously called ADAM2 or PH30) (Cho et al., 2000; van Gestel et al., 2007) and ß 1,4 galactosyltransferase I (GalTase) (Rodeheffer and Shur 2004, Shur et al., 2006). Interestingly, GalTase is so far the only primary zona binding protein of the sperm surface that is linked to the zona-induced acrosome reaction, which is part of primary zona binding cascade (for a recent review on the zona induced acrosome reaction, see Litscher et al., 2009). GalTase has been reported to act with heterotrimeric G proteins and to cationic channels in order to execute the acrosome reaction (for a model, see Shur et al., 2006). Indeed the acrosome reaction is induced by primary zona binding and signal transduction over the adhered plasma membrane is required for stimulation of this Ca^{2+} driven multiple fusion event. GalTase probably requires additional (and species specific) factors for primary zona binding. One such factor is SED-1 (also called P47), a secretory protein that becomes peripherally associated to the sperm's surface in the epididymis. The interaction with GalTase has been demonstrated (Shur et al., 2006; Copland 2009, Ensslin and Shur 2003) and it is one of the major primary zona binding proteins in our direct binding assay (van Gestel et al., 2007). Other factors associated with the sperm surface include: (i) SPAM 1 (also known as PH20 or 2B1) which also has an epididymal origin but becomes associated to the sperm surface via a glycosylphosphatidylinositol anchored covalent linkage (Morin et al., 2010, Thaler and Cardullo 1995, Seaton et al., 2000, Sleight et al., 2005) although it is also reported to be abundantly expressed in the testis (Hou et al., 1996); (ii) an hyaluronan binding protein called HYBP1, which binds with intrinsic clustered mannose residues to the zona pellucida of ruminants (Ghosh and Datta 2003) and originates at the surface of elongating spermatids in the testis; (iii) Other carbohydrate modifying enzymes associated to the sperm surface are also involved in primary zona binding including *N*-acetylglucosaminidase (Zitta et al., 2006); (iv) fucosyltransferease (Chiu et al., 2007) and (v) arylsulfatase (likely, to originate from seminal vesicles Gadella et al., 1993; for review see Tanphaichitr et al., 2007); (vi) The enzyme carbonyl reductase is secreted and associates to sperm in the epididymis and has been reported to be involved in zona binding in the hamster (Montfort et al., 2002) and was found in our direct porcine zona binding assay (van Gestel et al., 2007); (vii) Similary AQN-3 (one of the porcine spermadhesins) is secreted by the epididymis, coats the sperm surface and remains associated to the isolated apical membrane fraction even under very stringent conditions and has high affinity for the zona pellucida (van Gestel et al., 2007); (viii) Another protein with firm peripheral association to the sperm surface after being secreted in the epididymis is CRISP1 (Busso et al., 2007); (ix) The interaction of sperm surface and acrosome oriented proteasomes with ubiquitinated zona proteins has been implied to be involved in sperm-zona binding (for review see Zimmerman & Sutovsky, 2009). Despite the convincing data set that emerged from this group, their finding requires further investigation since both proteasomes and poly-ubiquitin tags to proteins (recognition signal for the proteasome for protein binding and destruction) are supposed to be restricted to the cytosol (Gallastegui and Groll 2010). Therefore, the reported topologies of proteasomes at the sperm surface and

in the lumen of the acrosome and of the polyubiquitin tags at the zona pellucida, which is an extracellular matrix, are both unexpected. (x) Other proteases (Deppe et al., 2008, 2010); and (xi) dipeptidases (Deguchi et al., 2007) have been implied to play a role in zona binding and their activity is partly regulated by the steroids progesterone and estradiol, of which is known (also in ruminants) that their levels are enhanced after ovulation at the period when the acrosome reaction at the zona pellucida takes place in vivo (Lukoseviciute et al., 2007).

Secondary zona binding

After the acrosome reaction a new layer of molecules is exposed for the so called secondary zona binding. The intra-acrosomal zona binding proteins become exposed to the zona pellucida and bind to ZP2. Most of the acrosome content is not released from the acrosome but remains associated with each other in a kind of enzyme matrix (Kim and Gerton 2003).The enzymes are slowly released during sperm zona drilling. The enzyme matrix should have two functionalities namely it should have affinity for ZP2 in the zona pellucida (the drilling sperm with high lateral head displacement properties need to remain attached to the zona pellucida) and the enzyme matrix needs to functionally dissociate the 3D matrix of the zona proteins in order to pave the way for the sperm towards the perivitelline space. Most likely the kinetics of acrosome content release and composition is highly species specific.

Classically the enzyme acrosin has been found to serve the two tasks required for secondary zona binding namely to bind to ZP2 and to cleave proteins as a serine protease (Dunbar et al., 1985) but its function in this process has been questioned as acrosin knock out mice were fertile (Crosby and Barros, 1999) However, considering the complexity of sperm-zona binding in which from the sperm's side multiple proteins are involved, it is possible that redundancy of individual proteins may explain why knock out phenotypes are capable of fertilization. Without redundancy the knock out genotype probably under evolutionary pressure will be less efficient as sperm with a full set of zona binding proteins (see Gadella et al., 2008). A reduced complement may be sufficient for fertilization, but a full complement will provide the sperm with more capability for fertilization. Sperm-zona interactions functionally are selective and create a time delay for sperm to become adjacent to the oolemma. If a sperm is better suited to be the first to bind/fuse with the oolemma it will win the race and after generations that phenotype that maybe referred for generating offspring. In other words, simple knock out mice with a single protein deficiency may still show a fertile phenotype despite the fact the depleted protein has a zona binding function. Another intra-acrosomal protein involved in secondary zona binding and zona penetration is zonadhesin (Lea et al., 2001; Bi et al., 2003) and knock out mice lacking expression of zonadhesin were infertile with impaired sperm zona interactions (Tardif et al., 2010a). Zonadhesin appears to function in a species specific manner (Tardif et al. 2010b), which may be the reason why this protein is under such extreme fast molecular evolution (Herlyn and Zischler 2008) although the latter is also described for acrosin (Raterman and Springer 2008).

Other proteins reported to have secondary zona binding properties include the following: (i) intra acrosomal membrane protein 38 (IAM38 also known as sp38; Mori et al. 1993,1995, Yu et al., 2006), which also has a function in sperm penetration through the zona pellucida; (ii) SAMP14, an acrosomal membrane-associated, glycosylphosphatidylinositol-anchored member of the Ly-6/urokinase-type plasminogen activator receptor superfamily (Shetty et al., 2003); (iii) SAMP32 which is associated with the acrosomal membrane is involved in secondary zona binding and zona penetration (Hao et al., 2002); and (iv) SPAG9 which has a structural homology with c-Jun N terminal kinase interacting protein 3 and is an intra-acrosomal protein with zona

affinity and thus can be considered as a secondary zona binding protein (Jagadish et al., 2005).

Remarkably a number of primary zona binding proteins (localized at the sperm surface) are also present in the acrosome often as acrosome specific isoforms. An example of this is SPAM-1, which is 80 kDa in the acrosome (from testicular origin) and at the C-terminus 10 kDa longer than the epidiymal iosoform present at the sperm surface (Morin et al., 2010). Other examples are hyaluronidase and PH20 that are localized in the acrosome and on the sperm surface, respectively (Thaler and Cardullo 1995; Overstreet et al., 1995).

Although secondary zona binding has been postulated to be ZP2 specific, the intra-acrosomal protein sp56 has specific affinity for ZP3 (Buffone et al., 2008a,b). In fact, sp56 was originally believed to be a sperm surface protein, which turned out to be incorrect (for review see Wassarman 2009). It is of interest that sp56 is released from the acrosome as a smaller processed protein with no affinity for the unfertilized zona pellucida (Buffone et al., 2009b). In order for sperm to penetrate the zona pellucida, there must be a mechanism that permits binding of the zona proteins followed by opening of the zona pellucida matrix and renewed binding. If so, the sp56 protein should stop interacting with ZP3 and release of the cleaved form of sp56 might permit renewed binding of intact sp56. The dogma that secondary zona binding is strictly ZP2 specific perhaps needs to be reconsidered. The alternative explanation provides a model in which a sort of preliminary diffusion of sp56 from the acrosome to the sperm surface takes place prior to zona binding (Wassarman 2009; Figure 2). Likewise, the unique role of ZP2 for secondary sperm-zona binding has recently been challenged as the uncleaved form of ZP2 turned out to be required for primary zona binding (Gahlay et al., 2010; Figure 2).

Beyond the protease activity of acrosin and the hyaluronidase activity released from the acrosome during the acrosome reaction, it is not clear what type of modifications are made by the acrosome enzymes to allow sperm penetration through the zona pellucida. Interestingly, relatively high titers of ß *N*-acetylhexosaminidase, ß galactosidase, and ß mannosidase were found in the acrosome of porcine and bovine sperm (Hayashi et al., 2004). Thus, beyond secondary zona binding proteins, specific carbohydrate modifying enzymes might be involved in paving the way for sperm to progress in the voyage towards the oolemma and enable zona penetration (Miller et al., 1993).

Oviduct secretions, follicular fluid and cumulus expansion

Due to the fact that most sperm-zona binding assays are based on preparations derived from epididymal or ejaculated sperm and collected zona pellucida material obtained from washed oocytes derived from follicles, the role of the oviduct, the follicular fluid and the cumulus expansion on the sperm surface have largely been neglected. In vivo the oviduct is the main site of sperm capacitation and has been been an area of intense investigation. Sperm cells entering the oviduct first interact with the oviduct epithelium and after a while are released in the capacitated state (for review on sperm-oviduct binding see Suarez 2008). It is clear that the oviduct can function as an active secretory organ resulting in the release of sperm from the oviduct epithelium (Sostaric et al., 2008). Although these secretions interact with the zona pellucida (see that section), they also interact with the sperm surface and thus could modulate primary sperm-zona binding in two ways. One example is the Glucose-regulated protein 78 (Grp78/BiP), which is secreted by human oviduct epithelial cells and modulates sperm-zona pellucida binding after its association to sperm cells (Marín-Briggiler et al., 2010). The oviduct may also serve to select sperm that have superior properties to fertilize (Gualteri and Talevi 2003) by binding and selectively releasing capacitated sperm (review for porcine and bovine species see Talevi and Gulatieri 2010). In buffalo the mannosylation of the zona pellucida (in part performed by the oviduct) has been found to be important for establishing interactions with the sperm surface

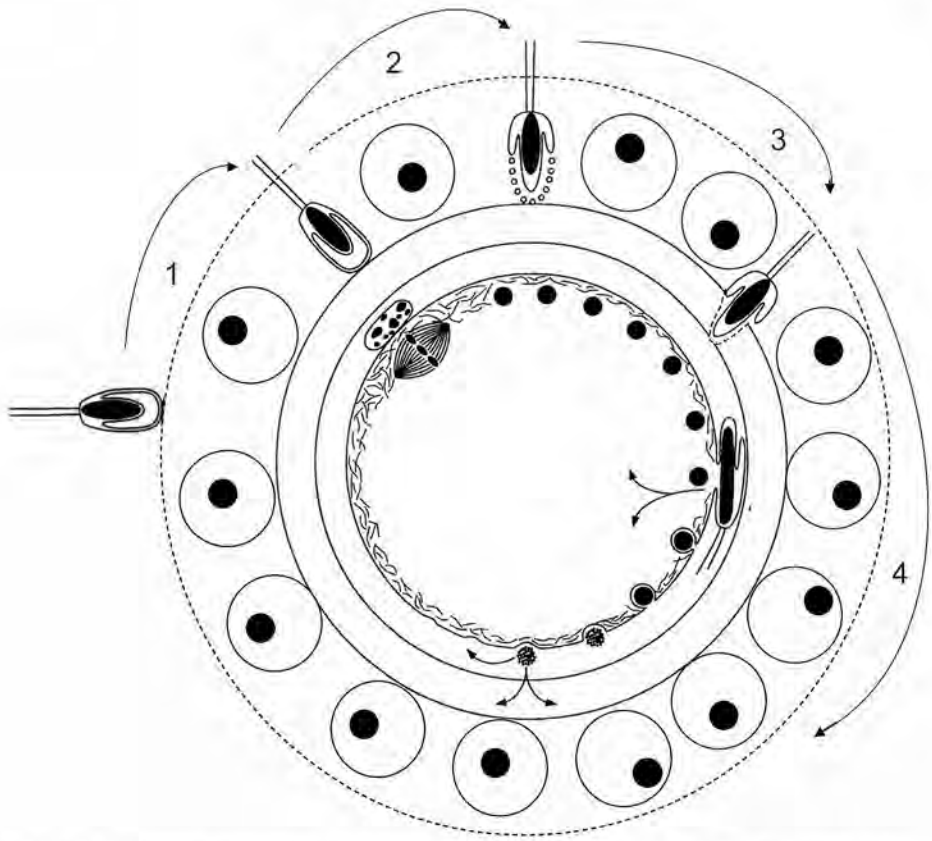

Fig. 2. The proposed sequence of events around fertilization that relate to sperm-zona interactions. Sperm that have entered the oviduct will shed off decapacitation factors that were adhered peripherally to the sperm surface. During this process the acrosome is docked to the sperm plasma membrane and at the docked area the formation of high affinity zona binding complexes are formed (Tsai et al., 2010; van Gestel et al., 2007). **1.** It is not clear whether the resulting sperm which can associate with the extra cellular matrix of the expanded cumulus mass (here represented as one cell layer but in reality composed of more cell layers) surrounding the unfertilized oocyte induces some early steps of acrosomal fusion (proposed by Wassarman 2009) or that acrosome intact sperm are penetrating through the cumulus by the use of hyperactivated motility in combination with surface proteins (Forman and Ducibella 2006). Oviductal secreted proteins are also reported to be important for cumulus and zona pellucida properties (Avilés et al., 2010). **2.** The recognition of the zona pellucida (primary zona binding to ZP3/ZP4) and subsequent inititation of the acrosome reaction (or of the acute secretory phase of it, see point 1) which is induced by the zona pellucida. **3.** The acrosome reaction causes local modifications of zona proteins and the hyperactivated sperm can penetrate this structure due to secondary zona binding (to ZP2). The surface of the penetrating sperm will be further remodeled and this probably serves to enable the fertilization fusion (Tsai and Gadella 2009). **4.** Immediately after the fertilization fusion the cortical reaction causes an overall coating of the oolemma as well as the hardening of the zona pellucida by chemically altering zona proteins. The cleavage of ZP2 appears to be particularly instrumental for the release of sperm from the zona pullicida and to elicit an efficient block to polyspermy (Galthay et al, 2010).

hyaluran binding proteins (HABP-1) and thus for enhancing primary sperm-zona binding (Ghosh and Datta, 2003). It is possible that other non-testicular secretory proteins (for instance those described from the epididymis), known to be involved in primary sperm-zona binding are also secreted by the oviduct and recruited on the sperm surface in order to enhance primary zona binding.

Different glycosylated forms of the glycoproteins glycodelin (previously known as placental protein 14 or progesterone-associated endometrial protein) are present in the oviduct fluid, follicular fluid, and in the cumulus cell layer surrounding the zona pellucida (Yeung et al., 2007). A redistribution and different composition as well as reassociation of three different glycodelin isoforms were noted to occur in the oviduct, by follicular fluid and by the expanded cumulus. This occurs when sperm are progressing towards the zona pellucida and is reported to modulate sperm-zona binding and the zona-induced acrosome reaction (Chiu et al., 2003, 2007, Yeung et al. 2009). For a recent report on the effects of human oviduct fluid on sperm functioning with special attention to zona binding characteristics see Munuce et al., 2009.

Zona contraceptives

Porcine zona pellucida (pZP) can be purified in large quantities (van Gestel et al. 2007). When conjugated to an adjuvant, it can be used to immunize females towards porcine zona proteins (Choudhury et al., 2007). This approach has been used in swine but has also successfully been used across a variety of mammalian species. Currently, pZP vaccination is used to reduce fertility of wildlife populations (Fayrer-Hoskin 2008). However, it is not yet clear how this pZP vaccination works as acontraceptive. One of the possible mechanisms is that anti pZP antibodies block or alter the assembly of the zona pellucida during oocyte maturation. The other possibility is pZP antibodies bind to their epitopes on the normally formed ZP structure. If this epitope is functionally blocking the primary and/or secondary receptor (ZP3/ZP4 and/or ZP2), this will as a consequence prevent sperm recognition, acrosome reaction and/or zona penetration. However, one of the side effects of pZP vaccination is the induction of ovarian failure probably resulting in blocking oocyte development especially at the level of zona pellucida synthesis (Koyama et al., 2005).

Dogmas and new insights

Some dogmas on sperm-zona interactions still prevail in the literature. Several have been mentioned in this overview (see also Figure 2). However, the following dogmas are well accepted: (i) Originally, acrosome intact sperm were supposed to bind to the zona pellucida. On the other hand, the expanded cumulus has the capacity to induce the acrosome reaction (Mattioli et al., 1998) by interacting with a sperm surface glycosylphosphatidylionositol anchored protein (Yin et al., 2009). (ii) Primary sperm zona binding is believed to be ZP3 specific. However, sp56 an intra-acrosomal protein interacts specifically with ZP3 (Buffone et al., 2008a) and although alternative explanations can be given, it probably indicates that also other ZP proteins are involved in secondary sperm-zona binding. (iii) Zona binding is not sufficient to elicit the acrosome reaction and initial zona penetration, which is required to activate a mechano-sensory signal transduction system to evoke the acrosome reaction (Baibakov et al., 2007). How this relates to item (i) remains to be elucidated but it is possible that the premature acrosome reaction is initiated at the cumulus level as proposed by Wassarman (2009), and that subsequent binding to ZP3/ZP4 further induces full acrosome reaction. (iv) Our recently published findings showed that in vitro capacitation not only leads to enhanced zona binding affinity of acrosome intact sperm but also to the stable docking of the outer acrosomal membrane with the apical sperm plasma membrane (Tsai et al., 2010). This could indicate that the zona pellucida binding is required for the induction of the acrosome reaction. (v) Sperm cells that penetrate the cumulus

during in vivo fertilization conditions are acrosome intact and probably use sperm surface-associated components for the dispersal of the cumulus mass to ensure sperm penetration (especially PH-20 the GPI anchored form of hyaluronidase which is believed to be responsible, for review see Florman and Ducibella 2006). Effective zona drilling is therefore believed to be achieved only by sperm that were acrosome intact prior to zona binding and premature acrosome reacted sperm will not reach the zona pellucida. (vi) The recent finding on how zona proteins are secreted and folded by the oocyte and which domains and structures are regulatory has shed light on how the zona pellucida is structurally regulated (Monné et al., 2008). These new findings should be placed into the classical concept that ZP1 is the dimeric crosslinker of ZP2/ZP3 hetero-oligomeric filaments. In ruminant and porcine, zona pellucida ZP1, is absent but still a functional 7 um thick zona pelliucida matrix is formed. Probably, the postulated functions of the discrete zona proteins forming the zona pellucida defined earlier need some revisions and the individual properties of each zona protein may turn out to be shared by others. In the review of Florman and Ducibella (2006), alternative cross linking to form the three dimensional zona structure for the human zona pellucida has been depicted. (vii) The uncleaved form of ZP2 is also essential for primary sperm-zona binding in the mouse, and the cortical reaction induces the release of sperm by cleaving ZP2 (Gahlay et al., 2010). It is not clear why in porcine and bovine species a part of ZP2 is already cleaved prior to fertilization and how this relates to in vivo and in vitro control of a block to polyspermy. Moreover, it is also not known how pre- and post-fertilization modifications of zona proteins relate to each other (for instance the cleavage of zona proteins by released proteins from the acrosome when compared with released proteins from cortical granules).

(viii) Lastly, the extremely complicated glycosylation patterns of all zona proteins (see figure 1) and their relevance for the zona structure, sperm binding, acrosome reaction induction, sperm-zona penetration as well as the regulation of polyspermy blockage after the cortical reaction needs to be studied in further detail. To this end, upcoming sperm surface and zona pellucida proteomic approaches will not be sufficient (Brewis and Gadella 2010). Experts from glycobiological disciplines are required and glycomics technologies should be employed to reveal relevant structures and their three dimensional organization during sperm-zona interactions (Vanderschaege et al., 2010).

Author's note after acceptance of manuscript

In humans it has now been shown that ZP1 binds sperm (primary zona binding) and induces the acrosome reaction (Ganguly et al., 2010a) in a similar fashion as is mentioned for ZP4 in the review. Moreover, the glycosylation of ZP1 and a specific ZP domain in that protein are at least in part responsible for this bioactivity (Ganguly et al. 2010b). Therefore, the table in Figure 1 is incomplete as ZP1 has a role in primary zona binding of human sperm.

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