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More Than a Simple Lock and Key Mechanism: Unraveling the Intricacies of Sperm-Zona Pellucida Binding

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1. Introduction

Mammalian fertilization involves a concerted interplay between the male and female gametes that ultimately results in the creation of new life. However, despite the fundamental importance of gamete interaction, the precise molecular mechanisms that underpin and regulate this complex event remain to be fully elucidated. Such knowledge is crucial in our attempts to resolve the global problems of population control and infertility. The current world population has surpassed 7 billion people, and continues to grow at a rate of approximately 200 000 each day (UN, 2009). Alarming, the majority of this population growth is occurring in developing nations, and is driven in part by an unmet need for effective and accessible contraceptive technologies. Indeed, a recent study by the Global Health Council revealed that of the 205 million pregnancies recorded worldwide each year, 60-80 million of these are deemed to be unplanned or unwanted (Guttmacher, 2007). These concerning statistics highlight the inadequacies of our current armory of contraceptives and demonstrate the need for the development of novel methods for fertility control. By virtue of its specificity and its ability to be suppressed in both males and females, sperm interaction with the outer vestments of the oocyte, a structure known as the zona pellucida (ZP), represents an attractive target for the development of novel contraceptives. However, the realization of such technologies is predicated on a thorough understanding of the molecular mechanisms that underpin this intricate binding event.

Such knowledge will also contribute to the development of novel diagnostic and therapeutic strategies for the paradoxical increase in male infertility that is being experienced by Western countries. Indeed, male infertility has become a distressingly common condition affecting at least 1 in 20 men of reproductive age (McLachlan and de Kretser, 2001). In a vast majority

(>80%) of infertile patients sufficient numbers of spermatozoa are produced to achieve fertilization, however the functionality of these cells has become compromised, making defective sperm function the largest single defined cause of human infertility (Hull, et al., 1985, Ombelet, et al., 1997). Biologically, a major cause of impaired sperm function is a failure of these cells to recognize the surface of the egg. Defective sperm- zona pellucida interactions is thus a major cause of fertilization failure *in vitro* and bioassays of sperm- zona pellucida interaction are able to predict male infertility *in vivo* with great accuracy (Arslan, et al., 2006).

In this review we explore our current understanding of the mechanisms that are responsible for sperm- zona pellucida interactions. Consideration is given to well-established paradigms of receptor-ligand binding with an emphasis on the emerging evidence for models involving the participation of multimeric receptor complexes and the maturation events that promote their assembly.

2. Sperm-zona pellucida interactions

2.1. The mammalian zona pellucida

The zona pellucida (ZP) is a porous extracellular matrix that surrounds the oocyte (Dunbar, et al., 1994, Wassarman and Litscher, 2008). In the most widely accepted models of gamete interaction, the zona pellucida plays a critical role in tethering spermatozoa, and inducing the release of their acrosomal contents (Bleil and Wassarman, 1983). Binding to the zona pellucida is a highly selective and carefully regulated process that serves as an inter-species barrier to fertilization by preventing adherence of non-homologous sperm to eggs (Hardy and Garbers, 1994).

Although all mammalian eggs are enclosed in a zona pellucida matrix, it's thickness (~1-25µm) and protein content (~1-10ng) varies considerably for eggs derived from different species (Wassarman, 1988). In mice, the zona pellucida comprises three major sulfated glycoproteins designated ZP1 (200kDa), ZP2 (120kDa) and ZP3 (83kDa). Current evidence suggests that these proteins assemble into a non-covalently linked structure comprising ZP2-ZP3 dimers that polymerize into filaments and are cross-linked by ZP1 (Greve and Wassarman, 1985, Wassarman and Mortillo, 1991). In addition to orthologues of the three mouse zona pellucida proteins [hZP1 (100kDa), hZP2 (75kDa) and hZP3 (55kDa)], the human zona pellucida comprises a fourth glycoprotein, hZP4 (65kDa) (Bauskin, et al., 1999, Lefievre, et al., 2004), which is thought to be dysfunctional in the mouse (Lefievre, et al., 2004). The biological significance of the increased complexity in the zona pellucida of humans awaits further investigation. Given that the mouse remains the most widely studied model for understanding sperm- zona pellucida interaction, this species will serve as the focus for the following discussion.

2.2. The biochemistry of sperm-zona pellucida recognition

Sperm- zona pellucida interaction encompasses a complex sequence of events that relies on each gamete having achieved an appropriate level of maturity. Spermatozoa that approach

the oocyte have undergone a behavioral and functional reprogramming event within the female reproductive tract, termed capacitation (see section 2.3.1.3), which ultimately endows the cells with the competence for fertilization. Notwithstanding recent evidence to the balance of evidence favors a model for sperm- zona pellucida interaction that involves three distinct stages: the first comprises primary binding of acrosome-intact spermatozoa to the zona pellucida, this is then followed by secondary binding of acrosome-reacted spermatozoa to the zona pellucida, and finally penetration of the acrosome-reacted sperm through the zona pellucida and into the perivitelline space (Florman and Storey, 1982, Inoue and Wolf, 1975, Saling, et al., 1979, Swenson and Dunbar, 1982).

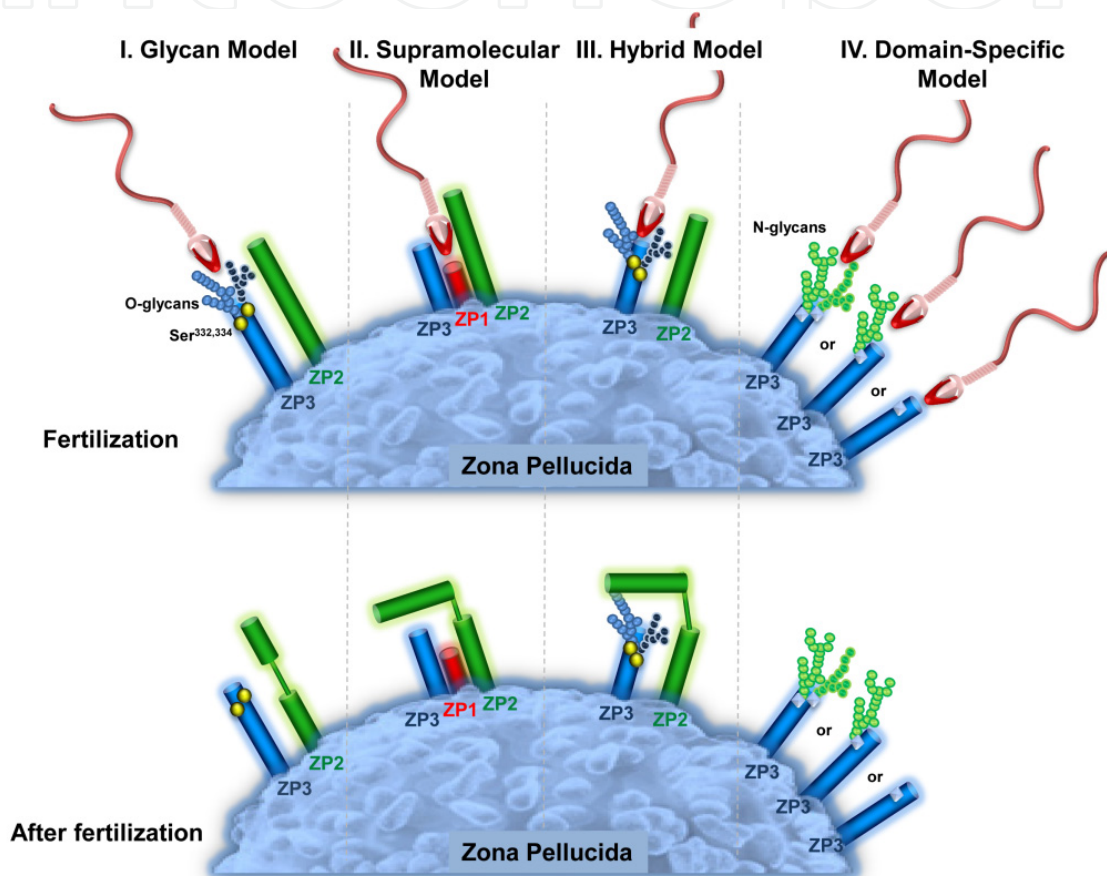


Figure 1. Putative models of sperm-zona pellucida binding. (i) The glycan model proposes that sperm binding is initiated via O-linked glycans that are attached at residues Ser³³² and Ser³³⁴ of ZP3. After fertilization, these residues are deglycosylated thereby preventing further sperm adhesion. (ii) The supramolecular structure model is based on the premise that the physical structure of the matrix formed by the three zona pellucida glycoproteins is critical for the binding of sperm. Following fertilization, ZP2 is processed in such a way that it prevents further sperm adhesion. (iii) The hybrid model incorporates aspects of both the glycan model and the supramolecular model and proposes that O-linked glycosylation is a critical determinant of sperm recognition. However, the key O-glycans reside on residues other than Ser³³² and Ser³³⁴. Furthermore, the modification of ZP2 that accompanies fertilization renders these O-glycans inaccessible to sperm. (iv) In contrast, the domain-specific model proposes that sperm bind with a variety of N-linked glycans attached to ZP3 and/or the peptide backbone of the glycoprotein depending upon its glycosylation status. The concepts proposed in this figure are adapted from those of Visconti and Florman, 2010 and Clark, 2010.

The initial stages of primary binding involve a relatively loose, non-species specific attachment that serves to tether spermatozoa to the surface of the oocyte (Schmell and Gulyas, 1980, Swenson and Dunbar, 1982). This weak binding is rapidly followed (within 10 minutes) by an irreversible tight binding event (Bleil and Wassarman, 1983, Hartmann, et al., 1972) that resists physical manipulation (Hartmann, et al., 1972, Inoue and Wolf, 1975) and is commonly species-specific. In the mouse, this latter event appears to involve binding of the spermatozoon to ZP3. This model emerged from early experiments performed by Bleil and Wassarman using crudely purified native zona pellucida that demonstrated that mouse ZP3 is responsible for acting as both a primary sperm ligand, preferentially binding the plasma membrane overlying the acrosome of acrosome-intact sperm, as well as an inducer of the acrosome reaction (Bleil and Wassarman, 1980a, Bleil and Wassarman, 1980b, Bleil and Wassarman, 1986, Vazquez, et al., 1989, Yanagimachi, 1994b). Purified mouse ZP3 was also shown to competitively inhibit binding of spermatozoa to homologous eggs *in vitro* (Bleil and Wassarman, 1980a, Endo, et al., 1987, Florman, et al., 1984, Florman and Wassarman, 1985, Leyton and Saling, 1989). The bioactive component of ZP3 responsible for mediation of sperm binding was initially traced to specific O-linked carbohydrate moieties that decorate the protein (Florman and Wassarman, 1985, Litscher, et al., 1995). In support of this model, complete deglycosylation, or selective removal of O-linked oligosaccharides eliminated the ability of ZP3 to interact with spermatozoa (Florman and Wassarman, 1985). In addition, the O-linked oligosaccharides released by these procedures were able to bind directly to spermatozoa and competitively inhibit their ability to adhere to the zona pellucida (Florman and Wassarman, 1985). Furthermore, genetically engineered chimeric mouse oocytes expressing human ZP3, acquire the same O-linked glycans as mouse ZP3 and bind mouse, rather than human, spermatozoa (Rankin et al., 1996; Hoodbhoy and Dean, 2004). Mutagenesis studies of ZP3 suggested that the key O-linked adhesion glycans for sperm are attached to either Ser³³² and/or Ser³³⁴ residues (Chen, et al., 1998) located within the C-terminal portion of the ZP3 polypeptide chain.

Notwithstanding such compelling evidence in favor of this classical model it has increasingly been drawn into question by a number of recent observations from genetically manipulated mouse models. For instance, female mice bearing targeted deletions of key glycosyltransferase enzymes responsible for the addition of O-linked glycans produce oocytes that display normal sperm binding characteristics (Ellies, et al., 1998). Furthermore, in a series of elegant experiments, transgenic mice have been produced in which the putative sperm binding residues were mutated (Ser^{329, 333, 334}→Ala, Ser³³¹→Val, Ser³³²→Gly) to eliminate potential O-linked glycosylation sites at Ser³³² and Ser³³⁴ (Gahlay et al. 2010). Females from these transgenic lines were shown to retain their fertility both *in vitro* and *in vivo*, and their oocytes maintained the ability to bind the same number of sperm as wild type mice, strongly suggesting that neither Ser³³² nor Ser³³⁴ are critical for sperm- zona pellucida recognition. The latter findings are perhaps best explained by detailed glycoproteomic analyses that have revealed Ser³³² and Ser³³⁴ are in fact unlikely to be glycosylated in mouse ZP3 (Boja et al. 2003; Chalabi et al. 2006).

These collective findings have led to the proposal of a number of alternative models of sperm- zona pellucida adhesion (Fig. 1), including the: (i) original glycan model that proposes the importance of O-linked glycosylation at Ser³³² and Ser³³⁴; (ii) a supramolecular structure model in which the sperm binding domain is formed by the complex of the three major zona pellucida glycoproteins and regulated by the cleavage status of ZP2 (Rankin et al. 2003), (iii) a hybrid model that incorporates elements of both former models by proposing that sperm bind to an O-glycan that is conjugated to ZP3 at a site other than Ser³³² or Ser³³⁴ (Visconti and Florman, 2010) and that sperm access to this glycan is regulated by the proteolytic cleavage state of ZP2; (iv) domain specific model that envisages a dual adhesion system in which sperm protein(s) interact with the glycans and/or the protein backbone of ZP3 depending on its glycosylation state (Clark, 2011) and (v) a novel model in which gamete recognition is able to be resolved into at least two distinct binding events, the first of which involves adherence to oviductal glycoproteins that are peripherally associated with the egg coat prior to engaging with a ZP3-dependent ligand (Lyng and Shur, 2009). The evidence in support of each of these models of gamete interaction has been reviewed in depth previously (Dean 2004; Clark 2010, 2011; Visconti and Florman, 2010). What is clear from these studies is that the initiation of gamete interaction is not mediated by a simple lock and key mechanism involving a single receptor-ligand interaction. Rather it is likely that sperm engage in multiple binding events with a variety of ligands within the zona pellucida matrix. An advantage of this complex adhesion system is that it would enhance the opportunities of sperm to bind to the oocyte and thus maximize the chance of fertilization. It may also account for the myriad of sperm receptors that have been implicated in this process (see below).

2.3. Sperm receptor molecules involved in zona pellucida interaction

2.3.1. Acquisition of the ability to engage in sperm-zona pellucida interactions

Prior to interaction with the egg, the sperm cell must undergo a complex, multifaceted process of functional maturation (Fig. 2). This process begins in the testes where spermatogonial stem cells are dramatically remodeled during spermatogenesis to produce one of the most highly differentiated and specialized cells in the body, the spermatozoon. After their initial morphological differentiation, these cells are released from the germinal epithelium of the testes in a functionally immature state, incapable of movement or any of the complex array of cellular interactions that are required for fertilization (Hermo, et al., 2010a). In all mammalian species, the acquisition of functional competence occurs progressively during the cells descent through the epididymis, a long convoluted tubule that connects the testis to the vas deferens (Fig. 2B). A remarkable feature of epididymal maturation is that this process is driven entirely by extrinsic factors in the complete absence of nuclear gene transcription and significant protein translation within the spermatozoa (Engel, et al., 1973). The surface and intracellular changes associated with epididymal maturation prepare the spermatozoa for their final phase of maturation within the female reproductive tract, whereby they realize their potential to bind to the zona pellucida and ultimately fertilize the egg (Bailey, 2010, Fraser, 2010, Yanagimachi, 1994a).

2.3.1.1. Spermatogenesis

Spermatogenesis describes the process by which spermatozoa develop from undifferentiated germ cells within the seminiferous tubules of the testis. It is characterized by three functional stages: proliferation, meiosis and metamorphosis. During the proliferation phase, spermatogonial germ cells undergo several mitotic divisions in order to renew themselves in addition to producing spermatocytes (Brinster, 2002, de Rooij, 2001, Dym, 1994, Oatley and Brinster, 2006). These cells then undergo two meiotic divisions to form haploid spermatids. The latter then develop into spermatozoa via an extremely complex process of cytodifferentiation and metamorphosis. This includes structural modifications to the shape of their nucleus, compaction of the nuclear chromatin, formation of an acrosomal vesicle and establishment of a flagellum allowing for the subsequent

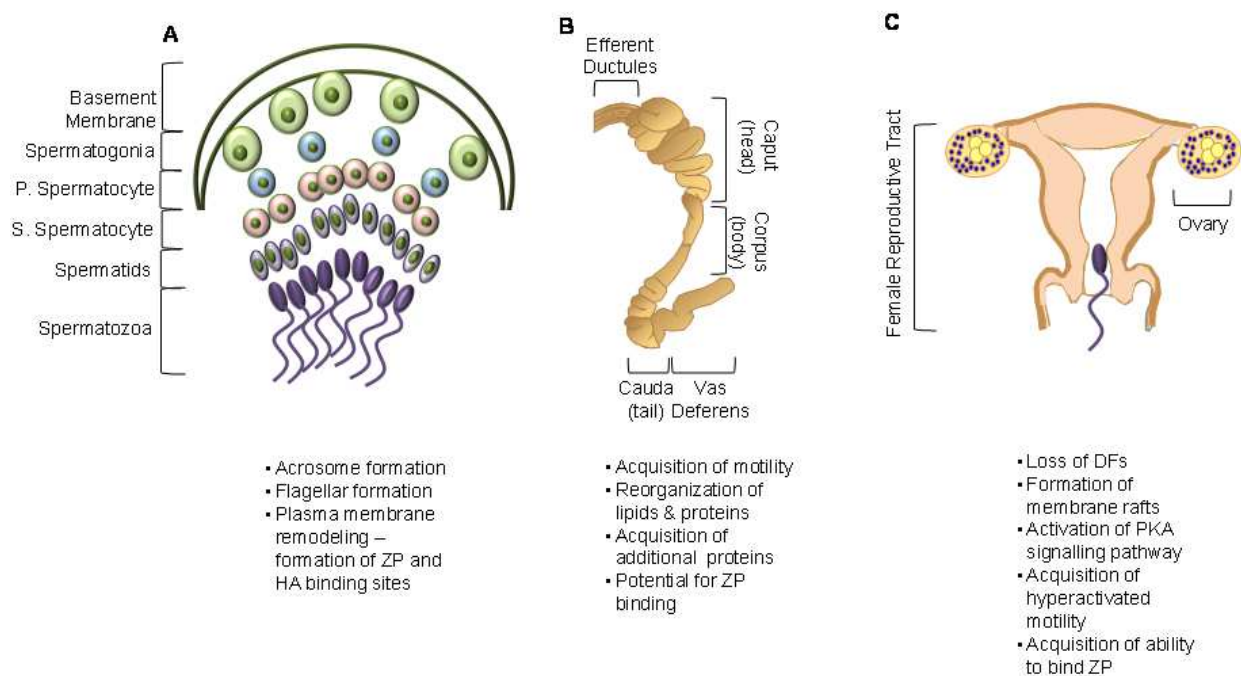


Figure 2. Acquisition of spermatozoa's ability to engage in interaction with the oocyte. (A)

During spermatogenesis, primordial germ cells undergo several phases of mitotic and meiotic divisions in order to produce morphologically mature but functionally incompetent spermatozoa. Of particular importance is the process of spermiogenesis, whereby spermatids undergo a process of cytodifferentiation that culminates in the production of spermatozoa. In the course of this dramatic transformation an acrosomal vesicle is formed in the anterior region of the sperm head and a flagellum develops posteriorly. The plasma membrane is also remodeled to produce zona pellucida (ZP) and hyaluronic acid (HA) binding sites. (B) Upon leaving the testis, spermatozoa traverse the epididymis and acquire the ability for forward progressive movement and to adhere to the zona pellucida surrounding the oocyte. These changes occur as a result of the reorganization of specific lipids and proteins. (C) However, it is not until the spermatozoa undergo a final phase of maturation termed 'capacitation' in the female reproductive tract that they realize the potential for zona pellucida interaction via the induction of hyperactivated motility along with the sequential loss of decapacitation factors (DFs), formation of membrane rafts and activation of key signaling cascades.

development of motility. The latter series of modifications that produce terminally differentiated spermatozoa from spermatids is referred to as spermiogenesis. Of particular importance to fertilization, is the formation of the acrosome during this stage. As seen by light microscopy, acrosomal development begins with the production of small proacrosome granules derived from the Golgi apparatus that lies adjacent to the early spermatid nucleus. These subsequently fuse together to form the acrosome, a large secretory vesicle that overlies the nucleus (Leblond and Clermont, 1952). There is also evidence to suggest that, in addition to the Golgi apparatus, the plasma membrane of the cell and endocytotic trafficking may also play a fundamental role in the formation of the this exocytotic vesicle (Ramalho-Santos, et al., 2001, West and Willison, 1996). Once formed, the acrosome remains associated with the nucleus of the spermatid, and subsequently the spermatozoa for the remainder of its life, and is of critical importance during fertilization due to its ability to aid in the penetration of the zona pellucida surrounding the ovulated oocyte. This function is, in turn, attributed to the hydrolytic enzymes enclosed within the acrosome. Notwithstanding recent evidence to the contrary, it is widely held that the release of these enzymes occurs upon engagement of sperm binding to the zona pellucida and facilitates localized digestion of the zona matrix, thereby facilitating sperm penetration through this barrier and providing access to the oocyte. The acrosomal enzymes are mostly derived from the lysosome, although several are unique to this organelle (Tulsiani, et al., 1998). In general terms, the acrosome can be divided into compartments, the first of which contains soluble proteins such as didpetididyl peptidase II and cystein-rich secretory protein 2 (Hardy, et al., 1991). The second compartment is known as the acrosomal matrix and contains the insoluble fraction of the enzymes including apexin (Kim, et al., 2001, Noland, et al., 1994, Westbrook-Case, et al., 1994), acrosin and acrosin-binding protein (Baba, et al., 1994b), and sp56, which has been previously implicated in the ability of sperm to interact with the zona pellucida (Buffone, et al., 2008a, Buffone, et al., 2008b).

In addition to the formation of the acrosome during spermiogenesis, the sperm develop a cytoplasmic droplet as well as undergoing plasma membrane remodeling events. The cytoplasmic droplet was first described by Retzius in 1909 as being a portion of germ cell cytoplasm that remains attached to the neck region of elongating spermatids. In most species, the cytoplasmic droplet migrates along the midpiece from the neck to annulus and is transiently retained by spermatozoa as they migrate through the epididymis (Cooper and Yeung, 2003), while in others it remains on the spermatozoa in the epididymis and is not shed until the time of ejaculation (Cooper, 2005, Harayama, et al., 1996, Kaplan, et al., 1984, Larsen, et al., 1980). The precise function of this residual cytoplasm remains elusive although its retention beyond ejaculation is associated with poor sperm function. For example, the cytoplasmic droplet on human spermatozoa is associated with poor sperm motility (Zini, et al., 1998), abnormal head and midpiece morphology (Gergely, et al., 1999, Gomez, et al., 1996, Huszar and Vigue, 1993), lower fertilizing capacity (Keating, et al., 1997) and reduced zona pellucida binding (Ergur, et al., 2002, Huszar, et al., 1994, Liu and Baker, 1992). The mechanism by which these abnormal sperm exhibit reduced function is attributed to disturbed membrane

remodeling (Huszar, et al., 1997) and higher extents of lipid peroxidation (Aitken, et al., 1994, Huszar, et al., 1994, Ollero, et al., 2000). The latter is most likely due to the high levels of ROS produced by the cytoplasmic droplet itself (Aitken, et al., 1994, Gil-Guzman, et al., 2001, Gomez, et al., 1996, Huszar and Vigue, 1993, Ollero, et al., 2000), combined with the enriched polyunsaturated fatty acids derived from the membrane of the droplet (Huszar and Vigue, 1993, Ollero, et al., 2000). The plasma membrane remodeling event involves the formation of zona pellucida binding sites via protein transport, which is thought to be mediated by the molecular chaperone, HSPA2. In agreement with the observations discussed above, immature human sperm that fail to express HSPA2 display cytoplasmic retention and reduced zona pellucida binding (Huszar, et al., 2000). The sperm also develop the machinery necessary for functional motility during spermiogenesis. As the acrosome grows at one pole of the nuclear surface of round spermatids, paired centrioles migrate to the opposite pole where they initiate the formation of the flagellum. The flagellum consists of a neck piece, a mid piece, a principle piece and an endpiece (Fawcett, 1975, Katz, 1991). The motility apparatus of the flagellum consists of a central axoneme of nine microtubular doublets arranged to form a cylinder around a central pair of single microtubules (Fawcett, 1975).

In combination, these fundamental changes in structure and biochemistry result in terminally differentiated, highly polarized and morphologically mature spermatozoa. However, despite this level of specialization the spermatozoa that leave the testis are functionally incompetent, as yet unable to move forward progressively, nor interact with the zona pellucida and fertilize the oocyte. They must first traverse the epididymis, a highly convoluted tubule adjacent to the testis, during which time they undergo further biochemical and biophysical changes.

2.3.1.2. *Epididymal maturation*

Upon leaving the testes, the first region of the epididymis that immature sperm encounter is that of the caput (head). Within this region, the sperm are concentrated by a mechanism of resorption that rapidly removes almost all the testicular fluid/proteins that enter the epididymis. As they leave this environment and enter the corpus (body) epididymis, sperm begin to acquire their motility and fertilizing ability. These attributes continue to develop as the sperm move through the corpus, and reach an optimum level as they reach the cauda (tail) region where they are stored in a quiescent state prior to ejaculation (Fig. 2B) (Cornwall, 2009, Gatti, et al., 2004). Ground breaking research performed in the 1960's and 1970's provided the first evidence that the epididymis played an active role for the epididymis in sperm development (Bedford, 1963, Bedford, 1965, Bedford, 1967, Bedford, 1968, Orgebin-Crist, 1967a, Orgebin-Crist, 1967b, Orgebin-Crist, 1968, Orgebin-Crist, 1969). Most importantly, it was discovered that if sperm were held in the testis via ligation of the epididymal duct, they were unable to develop the ability to fertilize an ovum, and as such their maturation is not an intrinsic property (Cooper and Orgebin-Crist, 1975, Cooper and Orgebin-Crist, 1977).

Consistent with this notion, sperm maturation within the epididymis is not under genomic control, since the cells enter the ductal system in a transcriptionally inactive state with limited biosynthetic capacity (Eddy, 2002). Any subsequent molecular changes must therefore be driven by the dynamic intraluminal milieu in which they are bathed as they transit the length of the epididymal tubule (Cooper, 1986). This epididymal microenvironment is characterized by dramatic sequential changes in its composition, a reflection of segment-specific gene expression (Dube, et al., 2007, Jelinsky, et al., 2007, Jervis and Robaire, 2001, Johnston, et al., 2007) and protein secretion (Dacheux, et al., 2006, Dacheux, et al., 2009, Guyonnet, et al., 2011, Nixon, et al., 2002, Syntin, et al., 1996). The unique physiological compartments established by this activity are thought to have evolved to not only to support the maturation of spermatozoa, but to also to provide protection for the vulnerable cells during their transport and prolonged storage.

It is well established that as sperm descend through the epididymis they acquire the potential for forward motility (reviewed (Amann, et al., 1993, Cooper, 1993, Moore and Akhondi, 1996, Soler, et al., 1994). This progressive motion not only allows the sperm to negotiate the female reproductive tract, but has also been suggested to play a role in penetration of the oocytes outer protective barriers, including the cumulus oophorus and the zona pellucida. To date, the mechanisms underlying the acquisition of forward motility by cauda epididymal sperm have not been completely elucidated. However, a number of potential contributing factors have been identified. On a biochemical level, proteins from caput epididymal sperm contain a greater number of sulfhydryl groups than disulfide bonds. Importantly, the oxidation of these sulfhydryl groups during epididymal transit is correlated with stabilization of flagella, as well as the promotion of protein tyrosine phosphorylation on specific sperm proteins involved in key signaling pathways (Calvin and Bedford, 1971, Cornwall, et al., 1988, Seligman, et al., 2004). Additionally, there is also recent evidence to suggest that sperm isolated from the caput epididymis possess the ability to become motile, but that this activity is suppressed through the action of the cannabinoid receptor CNR1, which upon engagement with its ligand, the endocannabinoid 2-arachidonoylglycerol, suppresses the capacity for motility (Cobellis, et al., 2010). Furthermore, changes in the luminal environment, as well as specific post-translational modification to sperm proteins have been shown to affect the motility status of these cells during their transit through the epididymis. In relation to the former, acidification of the luminal contents of the epididymis work to maintain sperm in an immotile state. This is finely regulated by epididymal clear cells which are capable of sensing a rise in luminal pH or bicarbonate concentrations via the sperm specific adenylyl cyclase (SACY)-dependent rise in cyclic-adenosinemonophosphate (cAMP) (Pastor-Soler, et al., 2003, Shum, et al., 2009). In terms of post-translational modifications, proteomic analyses of sperm proteins within the epididymis have identified a number of potential targets affected by changes in expression, disulfide bond status, proteolysis and alterations such as phosphorylation (Baker, et al., 2005). Finally, glycolysis plays an essential role as an energy pathway to fuel forward progressive movement in mouse spermatozoa. This is evidenced by the observation that male mice with genetic ablations of the sperm-specific forms of key glycolytic enzymes (glyceraldehydes 3-phosphate dehydrogenase S or phosphoglycerate kinase 2) are infertile or have very low fertility (Danshina, et al., 2010, Miki, et al., 2004). In part this can be explained by significantly decreased levels of ATP production (4 to 10-times lower than wildtype sperm) resulting in poor, or

sluggish motility. Furthermore, the spermatogenic cell-specific type 7 hexokinase that is present in mouse spermatozoa undergoes cleavage of disulfide bonds during epididymal transit, resulting in increased hexokinase activity which, in turn, has been causally associated with the initiation of sperm motility (Nakamura, et al., 2008). This indicates that specific structural changes to proteins during epididymal maturation have functional consequences, improving sperm competence for motility, and subsequently their ability to engage in fertilization.

In addition to the maturation of the motility apparatus, the acquisition of zona pellucida binding is also temporally associated with the exposure of spermatozoa to two distinct subsets of macromolecular structures in the epididymal lumen: the first being amorphous chaperone-laden 'dense bodies' (Asquith, et al., 2005) and the second being membrane bound prostasome-like particles known as epididymosomes (Saez, et al., 2003). It has been suggested that these epididymal granules facilitate the transfer of proteins to the sperm surface during their transit of the organ (Asquith, et al., 2005, Saez, et al., 2003, Yano, et al., 2010). This is in keeping with the demonstration that biotinylated proteins are able to be transferred between epididymosomes and the sperm surface (Saez, et al., 2003). At present it remains to be determined how this transfer is mediated and the number of cargo proteins that are delivered to the maturing spermatozoa in this manner. Nevertheless, a number of proteins have been shown to be acquired by the sperm during epididymal transit. A non-exhaustive list of these proteins include HE5/CD52 (Kirchhoff and Hale, 1996), members of the ADAM family (Girouard, et al., 2011, Oh, et al., 2009), SPAM1 (Zhang and Martin-Deleon, 2003) and other hyaluronidases (Frenette and Sullivan, 2001, Legare, et al., 1999), macrophage migration inhibitory factor (MIF) (Eickhoff, et al., 2001, Frenette, et al., 2003, Girouard, et al., 2011) as well as a number of enzymes including aldose reductase and sorbitol dehydrogenase (Frenette, et al., 2004, Frenette, et al., 2006, Kobayashi, et al., 2002, Thimon, et al., 2008). Collectively these proteins are believed to participate in the modification of the sperm biochemistry and surface architecture conferring the potential to engage in oocyte interactions.

2.3.1.3. *Capacitation*

Although spermatozoa acquire the potential to fertilize an egg within the epididymis, the expression of this functional competence is suppressed until their release from this environment at the moment of ejaculation. Indeed they must first spend a period of time within the female reproductive tract (Austin, 1952, Chang, 1951) during which they undergo the final phase of post-testicular maturation, a process known as capacitation. Capacitation is characterized by a series of biochemical and biophysical alterations to the cell including changes in intracellular pH, remodeling of the cell surface architecture, changes in motility patterns and initiation of complex signal transduction pathways. These events have been correlated with a dramatic global up-regulation of tyrosine phosphorylation across a number of key proteins. The ensuing activation of these target proteins has, in turn, been causally linked to the initiation of hyperactivated motility, ability to recognize and adhere to the zona pellucida, and the ability to undergo acrosomal exocytosis (Nixon, et al., 2007). For the purpose of this review, focus will be placed on the molecular mechanisms that culminate in the ability of the sperm to interact with the zona matrix. Furthermore, as this is a cell-surface mediated event, discussion will be centered on the capacitation-associated pathways that mediate sperm surface remodeling.

One of the more widely accepted sequences for mammalian capacitation begins with the loss of surface-inhibitory factors, known as decapacitation factors. These factors mostly originate in the epididymis and accessory organs, and their removal from non-capacitated spermatozoa results in a rapid increase in their fertilizing ability (Fraser, 1984). Furthermore, as capacitation is a reversible process, addition of these decapacitation factors into a population of capacitating spermatozoa potently suppress their ability to recognize and fertilize an oocyte (Fraser, et al., 1990). A number of candidates with potential decapacitation activity have been identified including: DF glycoprotein (Fraser, 1998), phosphatidylethanolamine binding protein 1 (PEB1) (Gibbons, et al., 2005, Nixon, et al., 2006), sperm antigen 36, CRISP1 and plasma membrane fatty acid binding protein (Nixon, et al., 2006) and NYD-SP27 (Bi, et al., 2009). Following the release of these decapacitation factors, spermatozoa experience a dramatic efflux of cholesterol from the plasma membrane (Davis, 1981). This efflux appears to be driven by active sequestration upon exposure of the spermatozoa to an environment rich in appropriate cholesterol sinks (Davis, et al., 1979, Langlais, et al., 1988, Visconti, et al., 1999), and accounts for a striking increase in membrane fluidity. Bovine serum albumin is commonly used within *in vitro* capacitating media as a cholesterol acceptor, although analogous acceptor(s) are believed to be present within the female reproductive tract. Indeed, studies of human follicular fluid have identified the presence high concentrations of albumin and other cholesterol sinks (Langlais, et al., 1988). Cholesterol efflux from the plasma membrane has also been correlated with an influx of bicarbonate ions (HCO_3^-) into the cell (Boatman and Robbins, 1991, Chen, et al., 2000, Garty and Salomon, 1987, Okamura, et al., 1985). In addition to its key role in initiation of critical signal transduction cascades, HCO_3^- has itself been shown to have a more direct role in sperm surface remodeling via stimulation of phospholipid scramblase activity (Gadella and Harrison, 2000, Gadella and Harrison, 2002). The ensuing random translocation of phospholipids between the outer and inner leaflets of the bilayer serves to disrupt the characteristic membrane asymmetry, (Flesch, et al., 2001a). This redistribution of phospholipids has been suggested to prime the sperm plasma membrane for cholesterol efflux, thus rendering the cell more 'fusogenic' and responsive to zona pellucida glycoproteins (Harrison and Gadella, 2005).

A further consequence of capacitation-associated cholesterol efflux is the formation of membrane rafts and/or the polarized coalescence of these microdomains and their protein cargo into the anterior region of the sperm head, the precise location that mediate zona pellucida binding (Fig. 3). Membrane rafts are generally defined as small, heterogeneous domains that serve to compartmentalize cellular processes (Pike, 2006), and regulate the distribution of membrane proteins, the activation of receptors and initiation of signaling cascades (Brown and London, 1998, Brown and London, 2000, Simons and Ikonen, 1997, Simons and Toomre, 2000). Membrane rafts are highly stable structures due to the inflexible steroid backbone of cholesterol (Martinez-Seara, et al., 2008) and are therefore extremely resistant to solubilization by a number of non-ionic detergents (Schuck, et al., 2003). As such they are often referred to as detergent-resistant membranes (DRMs). However despite their stability, rafts remain highly dynamic entities and have been observed to display considerable lateral movement in various cell types as a response

to physical stimuli or cellular activation events (Simons and Vaz, 2004). In sperm, membrane rafts have been identified by the presence of several somatic cell raft markers including G_{M1} gangliosides, flotillin and proteins that have raft affinity due to the presence of glycosylphosphatidylinositol (GPI) anchors, including CD59 and SPAM1 (Nixon, et al., 2009, Sleight, et al., 2005, van Gestel, et al., 2005). Notably, the spatial distribution of membrane rafts within the sperm membrane is dramatically influenced by the capacitation status of the cells. Indeed, the uniform localization of rafts characteristically observed in non-capacitated spermatozoa is replaced by a pattern of confinement within the peri-acrosomal region of the sperm head following the induction of capacitation (Boerke, et al., 2008, Nixon, et al., 2009, Shadan, et al., 2004). This particularly interesting finding raises the possibility that membrane rafts are of significance in coordinating the functional competence of spermatozoa (Bou Khalil, et al., 2006). In keeping with this notion, recent studies have shown isolated DRMs are capable of binding to the zona pellucida of homologous oocytes with a high degree of affinity and specificity (Bou Khalil, et al., 2006, Nixon, et al., 2009, Nixon, et al., 2011) and that these membrane fractions contain a number of key molecules that have been previously implicated in sperm-zona pellucida interactions (Bou Khalil, et al., 2006, Nixon, et al., 2009, Nixon, et al., 2011, Sleight, et al., 2005). Taken together, such findings encourage speculation that sperm membrane rafts may serve as platforms that act to spatially constrain key zona pellucida recognition molecules and deliver them to their site of action on the anterior region of the sperm head during capacitation (Nixon, et al., 2009, Nixon, et al., 2011). Consistent with this notion, elegant real time tracking studies have demonstrated that cholesterol efflux initiates diffusion (and possibly formation) of novel membrane raft-like structures containing zona-binding molecules over the acrosome of live spermatozoa. Furthermore, following head-to-head agglutination spermatozoa show contact-induced coalescence of G_{M1} gangliosides suggestive of a specific mechanosensitive response that concentrates important molecules to the appropriate site on the sperm surface to mediate zona binding (Jones, et al., 2010).

In addition to stimulating the loss of cholesterol from the plasma membrane, and promoting aggregation of membrane rafts, the elevation of intracellular HCO_3^- also activates a unique form of soluble adenylyl cyclase (SACY), which synthesizes cAMP from adenine triphosphate (ATP) (Aitken, et al., 1998, White and Aitken, 1989). Calcium has also been shown to coordinate with bicarbonate to stimulate SACY, although the precise mechanism that underpins this interaction remains to be elucidated (Carlson, et al., 2007, Litvin, et al., 2003). The importance of SACY has been demonstrated by the fact that sperm from *Sacy*-null male mice display limited motility (Esposito, et al., 2004). Furthermore, inhibition of SACY activity in wildtype mice results in the obstruction of capacitation-associated tyrosine phosphorylation and *in vitro* fertilization (Hess, et al., 2005). In addition to SACY, intracellular levels of cAMP are also regulated by cAMP phosphodiesterases (PDEs) that degrade cAMP to 5'-AMP (Fig. 3). The initial production of cAMP activates protein kinase A (PKA) through association with the regulatory subunits of the enzyme, promoting dissociation and activation of the catalytic subunits that in turn catalyze the phosphorylation of serine/threonine residues (Uner and Sakkas, 2003). Activation of PKA

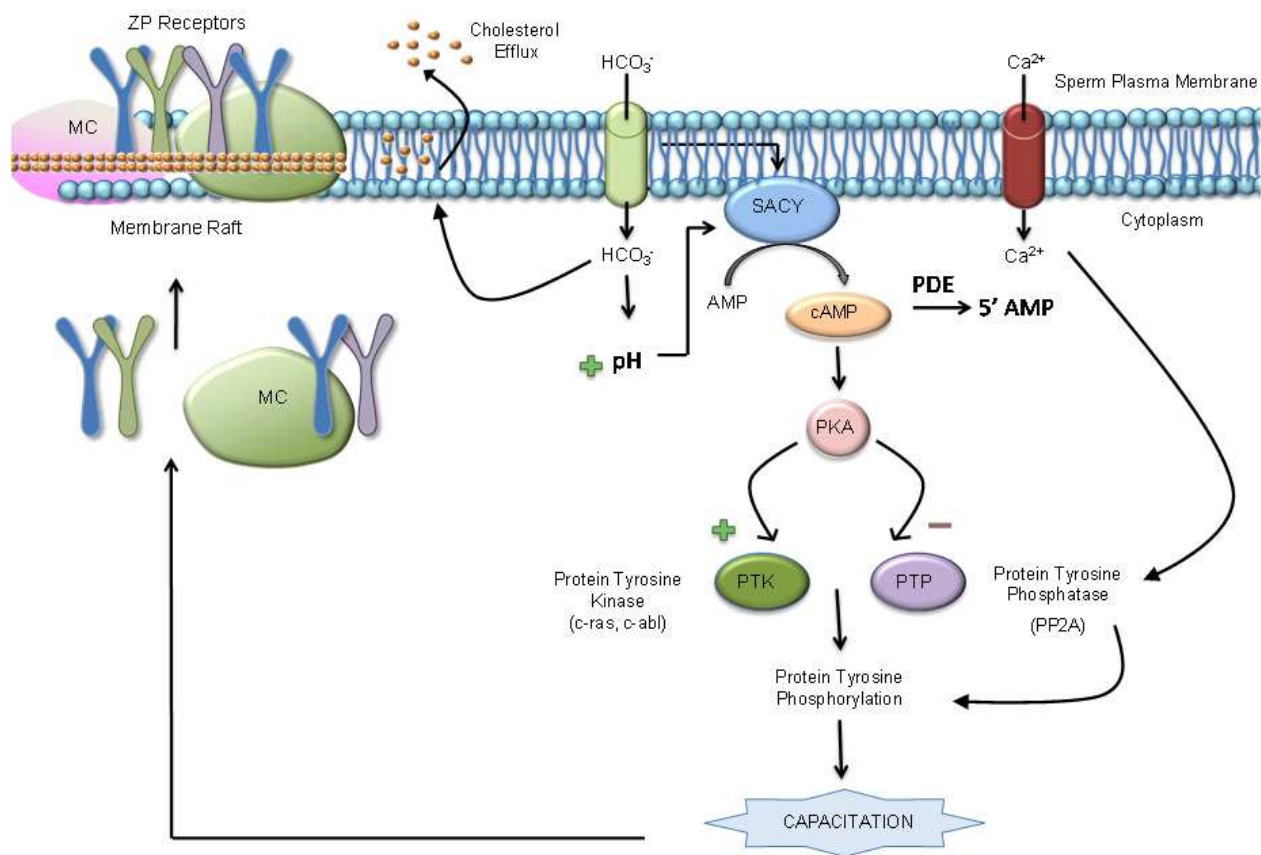


Figure 3. Model of mammalian sperm capacitation. Cholesterol efflux during the early phases of capacitation increases plasma membrane fluidity, facilitating the entry of bicarbonate (HCO_3^-) and calcium ions (Ca^{2+}) into the sperm cytosol through specific membrane channels. Cholesterol is preferentially lost from non-membrane raft portions of the plasma membrane, and appears to promote a polarized redistribution of membrane rafts to the anterior region of the sperm head. This event may serve to reposition key zona pellucida receptor molecules and enable their surface presentation and / or assembly into functional zona pellucida receptor complexes in this region of the sperm head. There is compelling evidence that such dramatic membrane remodeling events may be augmented by the action of molecular chaperones that are themselves activated during capacitation. This activation appears to be underpinned by a complex signaling cascade involving cross-talk between several pathways. In the most well characterized of these, a sperm specific form of soluble adenylyl cyclase (SACY) is activated by increases in intracellular bicarbonate, calcium and pH, leading to the production of the second messenger cyclic AMP (cAMP). cAMP, in turn, initiates the activation of protein kinase A (PKA), which then simultaneously inhibits the activity of protein tyrosine phosphatases (PTP) and activates protein tyrosine kinases (PTK). This dual regulation results in a global increase in protein tyrosine phosphorylation across a myriad of proteins, including a subset of molecular chaperones, and culminates in the functional activation of the cell. Calcium regulated adenylyl cyclases, phosphodiesterases (PDE), tyrosine kinases and tyrosine phosphatases have also been implicated in various aspects of capacitation associated cell signaling in the spermatozoa of a number of mammalian species.

also results in the induction of tyrosine phosphorylation across a number of substrates, most likely through activation of an intermediary protein tyrosine kinase (PTK) and/or inhibition of protein tyrosine phosphatases (PTP), or both. Of the potential candidates, inhibitory studies have implicated the promiscuous SRC kinase-family of PTKs in driving the increase in phosphotyrosine content (Baker, et al., 2006), especially in human spermatozoa (Lawson, et al., 2008, Mitchell, et al., 2008). However, more recent work has demonstrated that the suppression of capacitation-associated parameters induced by SRC kinase inhibitors is able to be overcome by incubation of sperm in the presence of Ser/Thr phosphatase inhibitors. In addition, sperm from *Src*-null mice contained similar levels of capacitation-associated tyrosine phosphorylation as wild-type sperm. These data indicate that SRC is not directly involved in capacitation-associated changes in tyrosine phosphorylation in mouse spermatozoa. They also provide evidence that capacitation is regulated by two parallel pathways, one requiring activation of PKA and another involving inactivation of Ser/Thr phosphatases, such as PP2A (Krapf, et al., 2010). Other potential candidates include c-ras which has been identified in human sperm (Naz, et al., 1992a), as well as c-abl which has been studied in both mouse (Baker, et al., 2009) and human models (Naz, 1998). It is important to note, that while the above canonical pathway is the primary pathway thought to induce capacitation, there is evidence to suggest that there is significant cross-talk with other signaling pathways. For instance, it has been demonstrated that a subset of the targets for capacitation associated protein tyrosine phosphorylation are activated by the extracellular signal-regulated kinase (ERK) module of the mitogen-activated protein kinase (MAPK) pathway. Interestingly, inhibition of several elements of this pathway results in suppression of sperm surface phosphotyrosine expression and a concomitant reduction in sperm-zona pellucida interactions (Nixon, et al., 2010).

Irrespective of the mechanisms, capacitation-associated tyrosine phosphorylation has been causally related to the induction of hyperactivated motility, increasing the ability of sperm to bind to the zona pellucida, priming of the cells for acrosomal exocytosis and ultimately enhancing their capacity to fertilize an oocyte (Leclerc, et al., 1997, Sakkas, et al., 2003, Urner and Sakkas, 2003, Visconti, et al., 1995b). The diversity of functions regulated by phosphorylation is consistent with the demonstration that this process occurs in a specific sequence within different compartments of the sperm cell, and is altered again upon binding to the zona pellucida (Sakkas, et al., 2003). In mouse spermatozoa, overt capacitation-associated increases in protein tyrosine phosphorylation have been documented in the flagellum, with principal piece phosphorylation preceding that of the midpiece. Several targets have been identified including aldolase A, NADH dehydrogenase, acrosin binding protein (sp32), proteasome subunit alpha type 6B, and voltage-dependent anion channel 2 among others (Arcelay, et al., 2008). In human spermatozoa however, this increase appears to be restricted to the principal piece, with evidence that both A-kinase anchor protein (AKAP) 3 and AKAP4 are targets (Ficarro, et al., 2003, Sakkas, et al., 2003). The tyrosine phosphorylation of proteins in the sperm flagellum has been causally related to the induction of hyperactivated motility (Mahony

and Gwathmey, 1999, Nassar, et al., 1999, Si and Okuno, 1999), a vigorous pattern of motility that is required for spermatozoa to penetrate through the cumulus cell layer and the zona pellucida in order to reach the inner membrane of the oocyte. In addition, to the increased phosphorylation, hyperactivation requires the alkalization of the sperm and is also calcium-dependent. The calcium required for the induction of hyperactivation can be mobilized into sperm from the external milieu by plasma membrane channel, and can also be released from intracellular stores, including the redundant nuclear envelope located at the base of the sperm flagellum, or the acrosome (Costello, et al., 2009, Herrick, et al., 2005, Ho and Suarez, 2003). Of particular importance in importing calcium into sperm are the CATSPER (cation channel, sperm associated) family of calcium channel proteins, which are sensitive to intracellular alkalization, and thus are critical for capacitation (Kirichok, et al., 2006, Lobley, et al., 2003, Qi, et al., 2007, Quill, et al., 2001, Ren, et al., 2001). Male mice null for each of the four individual *Catsper* genes have been shown to be infertile as they are incapable of the hyperactivated motility required for zona pellucida penetration (Carlson, et al., 2005, Jin, et al., 2007, Qi, et al., 2007, Quill, et al., 2001, Ren, et al., 2001).

In addition to the more widely studied phosphorylation of flagellum proteins, capacitation-associated increases in tyrosine phosphorylation have also been reported in an alternate set of proteins located in the sperm head (Asquith, et al., 2004, Flesch, et al., 2001b, Tesarik, et al., 1993, Urner, et al., 2001). Although these proteins represent only a minor proportion of the total pool of phosphorylation substrates in mouse spermatozoa, their importance has been highlighted by the observation that they are expressed on the surface of live, capacitated spermatozoa in a position compatible with a role in mediation of sperm-zona pellucida interactions (Asquith, et al., 2004, Piehler, et al., 2006). Furthermore, these phosphoproteins are present on virtually all sperm that are competent to adhere to the zona pellucida opposed to less than one quarter of sperm in the free swimming population. Although such findings invite speculation that a subset of proteins targeted for phosphotyrosine residues may directly participate sperm-zona pellucida adhesion, this conclusion is at odds with the fact that pre-incubation of sperm with anti-phosphotyrosine antibodies has no discernible effect on their subsequent fertilizing ability (Asquith, et al., 2004). Rather it has been suggested that, following their activation via phosphorylation, these proteins play an indirect role by mediating sperm surface remodeling to render cells competent to engage in zona pellucida adhesion (Fig. 3). In agreement with this proposal, a subset of phosphorylated proteins have been identified in the mouse as the molecular chaperone proteins heat shock protein (HSP) 60 (HSPD1) and endoplasmic (HSP90B1) (Asquith, et al., 2004). Such proteins have well-characterized roles in the folding and trafficking proteins, the assembly of multi-protein structures, and the translocation of proteins across membranes (Nixon, et al., 2005). In addition to mice, a similar cohort of molecular chaperone proteins have also been detected on the surface of sperm from other species including bull (Kamaruddin, et al., 2004), boar (Spinaci, et al., 2005) and human (Miller, et al., 1992, Naaby-Hansen and Herr, 2010), although their phosphorylation status in these species is less clear.

Maturational Phase	Changes contributing to acquisition of zona pellucida binding ability	References
Spermatogenesis	<ul style="list-style-type: none">• Primordial germ cells undergo multiple stages of mitotic and meiotic divisions, followed by a process of cytodifferentiation which results in a highly polarized cell• In early spermatids the Golgi apparatus is transformed into the acrosome• The flagellum is formed to provide sperm with the ability for forward progressive movement• Expression of the molecular chaperone in elongating spermatids is correlated with plasma membrane remodeling that results in the formation of zona pellucida and hyaluronic acid binding sites. These HA binding sites are thought to be responsible for the sperm to penetrate the cumulus cell layer surrounding the oocyte	(Berruti and Paiardi, 2011, Hermo, et al., 2010b, Hermo, et al., 2010c, Huszar, et al., 2007)
Epididymal Transit	<ul style="list-style-type: none">• Lipid architecture is remodeled in preparation for the formation of membrane rafts during capacitation• Protein architecture is altered. Existing proteins are unmasked or undergo post-translational modifications, or alternatively novel proteins are integrated into the plasma membrane via epididymosomes and intraluminal fluid• Motility machinery is matured in preparation for acquisition of motility• Upon reaching the cauda epididymis spermatozoa are capable of a sinusoidal movement pattern characterized by a symmetrical tail motion at high frequency and low amplitude• Increase in ability to recognize and interact with zona pellucida	(Cooper, 1986, Cooper and Orgebin-Crist, 1975, Dacheux and Paquignon, 1980, Jones, 1998, Jones, et al., 2007)
Capacitation	<ul style="list-style-type: none">• Loss of specific decapacitation factors (DFs) allows freshly ejaculated spermatozoa to commence capacitation• Cholesterol efflux from the plasma membrane increases membrane fluidity promoting lateral movement of integral proteins, as well as the formation of membrane rafts• Influx of HCO_3^- activates key signaling cascades whereby SACY stimulates cAMP and in turn PKA. This results in increased tyrosine phosphorylation of specific sperm proteins• In the tail, AKAPs become activated via this phosphorylation and induce a hyperactivated form of motility which allows the sperm to navigate through the oviduct to the site of ovulation.• Key zona pellucida recognition molecules aggregate to the apical region of the sperm head, using membrane rafts as a platform to mediate zona pellucida interaction	(Fraser, 1984, Jones, et al., 2010, Nixon, et al., 2009, Nixon, et al., 2011, Sleight, et al., 2005, Suarez, 2008, Visconti, et al., 1995a)

Table 1. Summary of specific biochemical- and biophysical-changes that occur during mammalian sperm maturation.

Although the precise role that these surface expressed chaperones play in preparing the sperm for their interaction with the oocyte remains to be established, one possibility is that they promote the presentation and/or assembly of oocyte receptor complex(es) on the sperm surface (Asquith, et al., 2004) (Fig. 3). This notion is supported by the observation that a subset of chaperones have been shown to be the subject of dynamic redistribution during capacitation, leading to their exposure on the anterior region of the sperm head (Asquith, et al., 2005, Dun, et al., 2011). Despite this relocation, a direct role for the chaperones in the mediation of sperm-zona pellucida interactions has been discounted on the basis that anti-chaperone antibodies consistently fail to compromise sperm-zona pellucida adhesion (Asquith, et al., 2005, Dun, et al., 2011, Walsh, et al., 2008). The chaperones do however form stable interactions with a number of putative zona pellucida adhesion molecules which, as discussed below (see Section 2.3.2), appears to indicate that they play an indirect role in gamete interaction. Whether a similar role extends to molecular chaperones in the spermatozoa of other species, such as our own, remains somewhat more controversial. A study by Mitchell *et al* (2007) failed to localize any of the prominent chaperones to the sperm surface, nor secure evidence for the capacitation-associated phosphorylation of these chaperone proteins (Mitchell, et al., 2007). However, a more recent study by Naaby-Hansen and Herr (2009) demonstrated the expression of seven members from four different chaperone families on the surface of human spermatozoa. They also demonstrated that inhibition of several isoforms of HSPA2 results in decreased fertilization rates *in vitro* (Naaby-Hansen and Herr, 2010). These studies are supported by earlier work which suggests that the absence of HSPA2 is correlated with decreased ability of sperm to bind to the zona pellucida (Huszar, et al., 2007).

2.3.2. Zona pellucida receptor candidates

Consistent with the apparent complexity of the zona pellucida ligands to which spermatozoa bind, a plethora of candidates have been proposed to act as primary receptors capable of interacting with the carbohydrate moieties and or protein present within the zona pellucida matrix. In most species the list is constantly being refined as new candidates emerge and others are disproven through, for example, the production of knockout models bearing targeted deletions of the putative receptors. Consistent with the notion that primary sperm- zona pellucida interaction involves engagement with specific carbohydrate structures on ZP3, a number of the identified sperm receptors possess lectin-like affinity for specific sugar residues (McLeskey, et al., 1998, Topfer-Petersen, 1999, Wassarman, 1992). In the mouse, the most widely studied model, these receptors include, but are not limited to: β -1,4-galactosyltransferase (GalT1) (Lopez, et al., 1985, Nixon, et al., 2001, Shur and Bennett, 1979, Shur and Hall, 1982a), ZP3R (or sp56) (Bookbinder, et al., 1995, Cheng, et al., 1994, Cohen and Wassarman, 2001), α -D-mannosidase (Cornwall, et al., 1991) and zonadhesin (Gao and Garbers, 1998, Tardif and Cormier, 2011, Topfer-Petersen, et al., 1998) (see Table 1). However, despite the wealth of knowledge accumulated about each of these putative zona pellucida receptors it is now apparent that none are uniquely capable of directing sperm- zona pellucida adhesion. For example, the targeted disruption of GalT1 in knockout

mice fails to result in infertility (Lu, et al., 1997). Although sperm from GalT1 null mice bind poorly to ZP3 and fail to undergo a zona-induced acrosome reaction, they retain the ability to bind to the ovulated egg coat *in vitro* (Lu and Shur, 1997). In a similar vein, a number of zona pellucida binding molecules have been identified in human spermatozoa, including sperm autoantigenic protein 17 (SPA17) (Grizzi, et al., 2003), fucosyltransferase 5 (FUT5) (Chiu, et al., 2003a, Chiu, et al., 2004), and mannose binding receptor (Rosano, et al., 2007). However, further analyses of these receptor molecules have compromised their status as being the single molecule responsible for zona pellucida interaction (see Table 2). In fact prevailing evidence now strongly suggests that no individual receptor is exclusively responsible for regulating gamete interaction. Underscoring the amazing complexity of this interaction, it has instead been proposed to rely on the coordinated action of several zona receptor molecules, which may be assembled into a functional multimeric complex.

Candidate (synonyms)	Species	Evidence	References
Angiotensin-converting enzyme (ACE)	Mouse Rat Horse Human	<ul style="list-style-type: none"> • Testis-specific form is found within developing spermatids and mature sperm • ACE KO mice are infertile due to defective transport in the oviducts as well as decreased zona pellucida binding • Play significant role in redistribution of ADAM3 to the sperm surface 	(Esther, et al., 1996, Foresta, et al., 1991, Kohn, et al., 1995, Langford, et al., 1993, Sibony, et al., 1993)
A disintegrin and metalloproteinase (ADAMs)	Mouse Rat Pig Human	<ul style="list-style-type: none"> • Family of transmembrane proteins that have varying roles in maturation of spermatozoa • ADAM3 has important role in zona pellucida binding • ADAM2 KO mice show strong suppression of zona pellucida binding and difficulty in moving through female reproductive tract, due to absence of ADAM3 in these mice • ADAM1a KO mice are fertile, but show decreased levels of ADAM3 on the sperm surface • ADAM1b KO mice are fertile 	(Kim, et al., 2004, Kim, et al., 2006a, Kim, et al., 2006b, Nishimura, et al., 2004, Nishimura, et al., 2007, Yamaguchi, et al., 2009)
α -D-mannosidase (MAN2B2)	Mouse Rat Hamster Human	<ul style="list-style-type: none"> • Integral plasma membrane protein that may facilitate sperm-zona pellucida binding by adhering to mannose-containing zona pellucida oligosaccharides 	(Cornwall, et al., 1991, Pereira, et al., 1998, Tulsiani, et al., 1993, Tulsiani, et al., 1989, Yoshida-Komiya, et al., 1999),

Candidate (synonyms)	Species	Evidence	References
		<ul style="list-style-type: none"> Pre-incubation of sperm with either D-mannose or anti-MAN2B2 antibody elicits a dose-dependent inhibition of zona pellucida binding 	
Arylsulfatase A (AS-A; ARSA)	Mouse Human Boar	<ul style="list-style-type: none"> Acquired onto the sperm surface during epididymal transit Addition of exogenous ARSA, or anti-ARSA antibodies inhibit zona pellucida binding in a dose-dependent manner ARSA-null males are fertile but fertility decreases with age 	(Carmona, et al., 2002, Hess, et al., 1996, Tantibhedhyangkul, et al., 2002, Weerachatanukul, et al., 2003)
Calmeglin (CLGN)/Calnexin/Calspernin (CALR3)	Mouse	<ul style="list-style-type: none"> CLGN- and CALR3-deficient mice are infertile due to defective sperm migration from uterus into the oviduct, as well as defective zona pellucidabinding CLGN is required for ADAM1a/ADAM2 dimerization CALR3 is required for ADAM3 maturation 	(Ikawa, et al., 2001, Ikawa, et al., 2011, Yamagata, et al., 2002)
GalT1 (β -1,4-galactosyltransferase; GALTase; GALT; B4GALT1)	Mouse Rat Human Guinea Pig Rabbit Bull Boar Stallion	<ul style="list-style-type: none"> Transmembrane protein located on the sperm head overlying the intact acrosome Transgenic mice overexpressing GalTase are hypersensitive to ZP3 and undergo precocious acrosome reactions Sperm from mice bearing targeted deletions in GalTase are unable to bind ZP3 or undergo ZP3-dependent acrosomal exocytosis GalTase-null sperm retain ability to bind to zona pellucida 	(Lopez, et al., 1985, Lopez and Shur, 1987, Shi, et al., 2004, Shur and Hall, 1982a, Shur and Hall, 1982b)
Fertilization antigen 1 (FA1)	Mouse Human Bull	<ul style="list-style-type: none"> Localized to the postacrosomal region of sperm head Anti-FA-1 antibodies have been implicated in immune infertility in humans No recorded knockout 	(Coonrod, et al., 1994, Menge, et al., 1999, Naz, et al., 1992b, Naz, et al., 1984, Naz and Zhu, 1998)

Candidate (synonyms)	Species	Evidence	References
Fucosyltransferase 5 (FUT5)	Human	<ul style="list-style-type: none"> Localized to the acrosomal region of the sperm head Pre-treatment of sperm with antibodies directed against FUT5 inhibits zona pellucida binding 	(Chiu, et al., 2003b, Chiu, et al., 2004)
Milk fat globule-EGF factor 8 (MFGE8; p47; SED1)	Mouse Boar	<ul style="list-style-type: none"> Protein is applied to the sperm acrosome during epididymal transit Binds specifically to the zona pellucida of unfertilized, but not fertilized eggs Recombinant MFGE8 and anti-MFGE8 antibodies competitively inhibits zona pellucida binding MFGE8 null males are subfertile and their sperm are unable to bind to the zona pellucida <i>in vitro</i> 	(Ensslin, et al., 1995, Ensslin and Shur, 2003)
Proacrosin (acrosin)	Mouse Boar	<ul style="list-style-type: none"> Localizes to acrosome and inner acrosomal membrane Mediates secondary zona pellucida binding via interaction with ZP2 Binding to zona pellucida is non-enzymatic and thought to involve recognition of polysulfate groups on zona pellucida glycoproteins Acrosin null males are fertile but display compromised zona pellucida penetration 	(Baba, et al., 1994a, Baba, et al., 1994b, Howes, et al., 2001, Howes and Jones, 2002, Moreno, et al., 1998, Urch and Patel, 1991)
Sperm adhesion molecule 1 (SPAM1; PH-20)	All mammals	<ul style="list-style-type: none"> Widely conserved sperm surface protein Localized to plasma membrane over anterior region of sperm head Possesses hyaluronidase activity that aids in the digestion of cumulus cells Relocalizes to inner acrosomal membrane following acrosome reaction; potentially participates in secondary zona pellucida binding SPAM1 null males are fertile although their sperm are less efficient in cumulus cell dispersal 	(Baba, et al., 2002, Hunnicutt, et al., 1996a, Hunnicutt, et al., 1996b, Lin, et al., 1994, Morales, et al., 2004, Myles and Primakoff, 1997)

Candidate (synonyms)	Species	Evidence	References
Sperm autoantigenic protein 17 (SPA17; SP17)	Mouse Rabbit Human Primates	<ul style="list-style-type: none"> Highly conserved protein localized to the acrosome and fibrous sheath Has been implicated in regulation of sperm maturation, capacitation, acrosomal exocytosis and zona pellucida binding Shown to bind to specific mannose components of the zona pellucida 	(Chiriva-Internati, et al., 2009, Grizzi, et al., 2003, Yamasaki, et al., 1995)
Spermadhesins (AWN; AQN-1; AQN-3)	Boar Stallion Bull	<ul style="list-style-type: none"> Are major components of seminal plasma May be involved in several sequential steps of fertilization through multifunctional ability to bind to carbohydrates, sulfated glycosaminoglycans, phospholipids and protease inhibitors 	(Petrunkina, et al., 2000, Sinowatz, et al., 1995, Topfer-Petersen, et al., 1998)
Sulfogalactosylglycerolipid (SGG)	Mouse Rat Human Boar	<ul style="list-style-type: none"> SGG is a major sperm sulfoglycolipid that putatively facilitates uptake of sulfolipid-immobilizing protein-1 (SLIP1) and ARSA Following capacitation, SGG is predominantly found in membrane rafts, microdomains that possess zona pellucida affinity Pre-incubation of sperm with monovalent anti-SGG Fab fragments significantly inhibits zona pellucida binding 	(Bou Khalil, et al., 2006, Kornblatt, 1979, Tanphaichitr, et al., 1990, Tanphaichitr, et al., 1993, Weerachatanukul, et al., 2001, White, et al., 2000)
Zonadhesin (ZAN)	Mouse Hamster Rabbit Boar Bull Horse Primates	<ul style="list-style-type: none"> Localizes to the apical region of the sperm head following spermatogenesis and epididymal maturation Features a mosaic protein architecture with several domains that potentially enable the protein to participate in multiple cell adhesion processes including zona pellucida binding 	(Bi, et al., 2003, Gasper and Swanson, 2006, Hardy and Garbers, 1994, Hardy and Garbers, 1995, Herlyn and Zischler, 2008, Hickox, et al., 2001, Olson, et al., 2004, Tardif, et al., 2010)

Candidate (synonyms)	Species	Evidence	References
		<ul style="list-style-type: none">• Appears to confer species specificity to sperm-zona pellucida adhesion in that sperm from <i>Zan</i> ^{-/-} males are able to bind promiscuously to the zona pellucida of non-homologous species	
ZP3R (sp56)	Mouse	<ul style="list-style-type: none">• Localized to the surface of the sperm head• Pre-incubation of sperm with anti-ZP3R antibodies blocks zona pellucida binding• Pre-treatment of sperm with recombinant ZP3R inhibits fertilization <i>in vivo</i>• EM localizes ZP3R within acrosomal matrix, but the protein appears to undergo a capacitation-associated relocation to the surface of the anterior region of the sperm head• <i>ZP3r</i> ^{-/-} males are fertile and their spermatozoa retain their ability to bind zonae of unfertilized eggs and undergo acrosomal exocytosis	(Hardy, et al., 2004, Muro, et al., 2012, Wassarman, 2009)

Table 2. Putative sperm- zona pellucida receptor candidates

2.4. Toward an integrated model of sperm- zona pellucida interaction

2.4.1. Multimeric protein complexes in zona pellucida binding

Despite decades of research, the specific molecular mechanisms that drive the initial interaction between the male and female gametes remain elusive. As stated previously, a myriad of diverse candidate molecules have been proposed as putative mediators of sperm binding to the zona matrix (Table 1). Regardless of this, prevailing evidence now indicates that none are uniquely responsible for directing or maintaining this interaction (Nixon, et al., 2007). Indeed, the classical model of a simple lock and key mechanism that prevailed in this field of research for several decades has been largely disproven. The fact that spermatozoa contain a multiplicity of zona pellucida receptor candidates allows for a level of functional redundancy commensurate with the overall importance of this fundamental cellular interaction. It also accounts for the succession of both low affinity and high affinity interactions (Thaler and Cardullo, 1996, Thaler and Cardullo, 2002) that characterize gamete interaction. Although the biochemical basis of this multifaceted adhesion process remains obscure, it is unlikely that it could be regulated by the activity of a single receptor.

Furthermore, mammalian spermatozoa undergo considerable changes in their already complex surface architecture during epididymal transit and the capacitation process in the female reproductive tract. Prior to these events, the cells are unable to recognize or bind to the zona pellucida. A simple lock and key mechanism involving a constitutively expressed surface receptor does not account for the need to undergo such radical alterations prior to obtaining affinity for the zona. Collectively, these data have led to an alternative hypothesis that sperm maturation leads to the surface expression and/or assembly of multimeric complex(es) compromising a multitude of zona pellucida receptors.

The concept that multimeric protein complexes are capable of regulating cell-cell interactions draws on an extensive body of literature. It is well known for instance that the human genome codes for in excess of 500 000 different proteins, of which an estimated 80% function as part of multimeric protein complexes, as opposed to individual proteins (Berggard, et al., 2007). In addition, there are many documented examples of cell-cell adhesion events that require the formation of multimeric protein complexes. As a case in point, β -catenin is well-known to form a complex with several other adhesion proteins, such as cadherin, at sites of cell-cell contact. Interestingly, the formation of these complexes is tightly regulated by phosphorylation and dephosphorylation of the N-terminus of β -catenin (Maher, et al., 2009). Tight junctions have also been shown to rely heavily on the formation of specific protein complexes, comprising transmembrane and membrane-associated proteins (Shen, et al., 2008). Studies with migrating cells, and other cell types that interact in fluid, dynamic environments similar to that in which gametes bind, have illustrated that they most likely rely on the sequential receptor-ligand interactions that are coordinated through the formation of protein adhesion complexes (Sackstein, 2005). In a situation analogous to that recorded in spermatozoa, recent work in cancer cell biology has described the importance of molecular chaperone complexes in increasing the migration and invasiveness of specific cancer types. Breast cancer in particular relies heavily on the action of HSP90 α in order to invade other cell types. In this case, HSP90 α is excreted by the cancer cell in order to act as a mediator between a complex of co-chaperones outside the cell, including HSP70, HSP40, Hop (HSP70/HSP90 organizing protein) and p23, subsequently activating MMP-2 (matrix metalloproteinase 2) (Eustace, et al., 2004, McCready, et al., 2010, Sims, et al., 2011). MMP-2 then acts to degrade proteins in the extracellular matrix of target cells, thus increasing the invasive ability of the malignant cancer cells (Folgueras, et al., 2004, Jezierska and Motyl, 2009).

The concept of a multimeric zona pellucida receptor complex in spermatozoa was originally proposed by Asquith *et al* in mouse spermatozoa (Asquith, et al., 2004). This work demonstrated the preferential tyrosine phosphorylation of a specific subset of molecular chaperones during capacitation. A finding that generated considerable interest was that this modification, coincided with the translocation of the chaperones the surface of the sperm head in the precise region that mediates zona pellucida binding. However, the failure of either anti-phosphotyrosine or anti-chaperone antibodies to compromise sperm- zona pellucida interactions led to the proposal that these chaperones may have an indirect role in zona pellucida interaction by virtue of their ability to coordinate the assembly of a zona

pellucida receptor complex during capacitation. A key observation in support of this model is that the chaperones, along with numerous putative zona pellucida receptors, partition into lipid microdomains or DRMs (discussed in Section 2.3.1.3). It is proposed that these microdomains may serve as platforms to recruit chaperone clients proteins and/or enhance productive interactions between these two classes of proteins (Nixon, et al., 2009). Indeed, independent evidence indicates that chaperones do form stable protein complexes within membrane rafts during the capacitation of mouse spermatozoa. For instance, Han *et al* (2011) have recently revealed that the molecular chaperones, HSPA5 and calnexin, associate with a number of client proteins to form a stable supramolecular complex on the surface of mouse spermatozoa. These client proteins include ADAM7 (a disintegrin and metalloprotease 7), a protease that is transferred to the sperm surface via epididymosomes as the cells transit through the epididymis (Oh et al. 2009) and belongs to a family of proteases that have been implicated in sperm migration in the female reproductive tract and adhesion to the zona pellucida (Muro and Okabe 2011) (Cho, et al., 1998, Shamsadin, et al., 1999, Yanagimachi, 2009). Interestingly the HSPA5/calnexin/ADAM7 complex resides within DRMs (membrane rafts) and its assembly is promoted by sperm capacitation (Han et al. 2011). In addition, recent work performed by Dun *et al* (2011) demonstrated the presence of a number of high molecular weight protein complexes expressed on the surface of capacitated mouse sperm utilizing the technique of Blue Native PAGE (Dun, et al., 2011). Of particular interest was the identification of the chaperonin-containing TCP-1 complex (CCT/TRiC) and its ability to form a stable complex with zona pellucida binding protein 2 (ZPBP2). In addition to independent evidence that ZPBP2 participates in zona pellucida binding (Lin, et al., 2007) the CCT/TRiC / ZPBP2 complex was also shown to display affinity for homologous zonae. Importantly, a complex of similar size and comprising the same combination of the CCT/TRiC / ZPBP2 complex was also recently identified via application of the same methodology in human spermatozoa and again shown to participate in zona pellucida interaction in this species (Redgrove, et al., 2011). The conservation of this complex implies that it may be involved in mediation of non-species specific initial interactions, which are relatively weak and forgiving of species barriers. The same may also be true of the 20S proteasome complex that has been shown to display a high level of conservation among the spermatozoa of different species. For instance, the proteasome complex has been described in the spermatozoa of pig, mouse and human and, in each of these species, it has been implicated in zonae interactions (Morales, et al., 2003, Pasten, et al., 2005, Yi, et al., 2010, Zimmerman, et al., 2011). Although this is a constitutively expressed complex, there is evidence that certain subunits of the complex may be subjected to post-translational modifications, including tyrosine phosphorylation, during capacitation (Redgrove et al., 2011). In this context it is noteworthy that the tyrosine phosphorylation of similar proteasome subunits has been shown to influence the substrate specificity of the complex in other cell types (Bose et al., 1999; Castano et al., 1996; Mason et al., 1996; Wehren et al., 1996). Taken together, these findings raise the possibility the proteasome complex may be activated during sperm maturation in preparation for its functional role(s) in sperm–oocyte interactions. These roles appear to extend beyond that of zona pellucida recognition (Zimmerman, et al., 2011) to include regulation of the acrosome reaction in addition to

penetration of the zona matrix (Kong, et al., 2009, Sutovsky, et al., 2004), (Morales, et al., 2003).

Interestingly, the indirect role of molecular chaperones in sperm- zona pellucida interactions appears to extend beyond the capacitation-associated remodeling of the sperm surface. Indeed, chaperones such as calmeglin, calsperrin, calnexin, and HSPA2 have been implicated in additional remodeling events during spermatogenesis and epididymal maturation. With respect to calsperrin and calmeglin, it has been shown that mice lacking these genes are incapable of binding to the zona pellucida, a defect that is attributable to the role these chaperones play in the maturation of ADAM3 (a protein required for fertilization), as well as the dimerization of an ADAM1 / ADAM2 heterodimer (Ikawa, et al., 2011). In contrast, calnexin has a primary role in retaining unfolded or unassembled N-linked glycoproteins in the ER (Sitia and Braakman, 2003). Importantly however, calnexin has also been shown to be present on the surface of mouse spermatozoa where it partitions into membrane rafts (Nixon, et al., 2009, Stein, et al., 2006). In addition to these lectin-like chaperones, testis-specific HSPA2 has been shown to be essential in several stages of spermatogenesis (Govin, et al., 2006) and, in the human, it has a prominent role in plasma membrane remodeling through the formation of zona pellucida and hyaluronic acid binding sites (Huszar, et al., 2007, Huszar, et al., 2006).

3. Summary

For decades, researchers have strived to find the key molecule on the sperm surface that is responsible for directing its binding to the zona pellucida in a cell and specifies specific manner. However, this premise of a simple lock and key mechanism has been increasingly drawn into question since it fails to account for the myriad of potential receptor molecules that have been identified over the intervening years and the fact that sperm- zona pellucida binding can be resolved into a number of sequential recognition events of varying affinity. Instead, owing largely to the application of elegant genetic manipulation strategies, it is now apparent that the interaction between the two gametes relies on an intricate interplay between a multitude of receptors and their complementary ligands, none of which are uniquely responsible. Such a level of functional redundancy is commensurate with the overall importance that this interaction holds in the initiation of a new life.

An important question that arises from this work is how the activity of such a diverse array of receptors is coordinated to ensure they are presented in the correct sequence to enable productive interactions with the zonae. One possibility is that the zona pellucida binding proteins are organized into functional receptor complexes that are assembled on the anterior region of the sperm head during the different phases of sperm maturation. Such a model may account for the need for the dramatic membrane remodeling events that accompany epididymal maturation and capacitation. Until recently a major challenge to this model has been the lack of direct evidence that sperm harbor multimeric protein complexes on their surface. However, through the application of a variety of novel techniques, independent laboratories have now verified that sperm do express high molecular weight protein

complexes on their surface, a subset of which possess affinity for homologous zonae. Furthermore, there is compelling evidence that the assembly and / or surface presentation of these complexes is regulated by the capacitation status of the cells (Dun, et al., 2011, Han, et al., 2010, Morales, et al., 2003, Redgrove, et al., 2011, Sutovsky, et al., 2004).

The conservation of complexes such as the 20S proteasome and CCT/TRiC implies that they are not involved in high-affinity species specific binding to homologous zonae. Rather they may mediate the initial loose tethering of sperm to the zona pellucida and / or downstream events in the fertilization cascade. It is therefore considered likely that the higher affinity, species-specific zona pellucida interactions that follow are executed by additional protein complexes that have been shown to reside in human and mouse spermatozoa (Dun, et al., 2011, Redgrove, et al., 2011) but have yet to be characterised. The proteomic profiling and functional characterization of these additional multiprotein complexes therefore promises to shed new light on the intricacies of sperm-egg interactions.

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