The function of chaperone proteins in the assemblage of protein complexes involved in gamete adhesion and fusion processes

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Abstract

The remarkable complexity of the molecular events governing adhesion and fusion of the male and female gametes is becoming apparent. Novel research suggests that these highly specific cellular interactions are facilitated by multiprotein complexes that are delivered to and/or assembled on the surface of the gametes by molecular chaperones in preparation for sperm–egg interaction. While the activation of these molecular chaperones and the mechanisms by which they shuttle proteins to the surface of the cell remain the subject of ongoing investigation, a compelling suggestion is that these processes are augmented by dynamic membrane microdomains or lipid rafts that migrate to the apical region of the sperm head after capacitation. Preliminary studies of the oocyte plasma membrane have also revealed the presence of lipid rafts comprising several molecular chaperones, raising the possibility that similar mechanisms may be involved in the activation of maternal fusion machinery and the regulation of oocyte plasma membrane integrity. Despite these findings, the analysis of oocyte surface multiprotein complexes is currently lacking. Further analyses of the intermediary proteins that facilitate the expression of key players in sperm–egg fusion are likely to deliver important insights into this unique event, which culminates in the cytoplasmic continuity of the male and female gametes.

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Introduction

Originally identified as inducible proteins involved in the protection of cells from multiple stresses, molecular chaperones are now recognized as participants in a diverse range of functions due to their ability to selectively bind to hydrophobic residues of target proteins, directing their involvement in correct protein folding or degradation pathways (Ellis 1987, Hendrick & Hartl 1993). The heat shock proteins (HSPs), among other ubiquitous chaperone families, have well-documented roles in preventing the aberrant association or aggregation of proteins in addition to facilitating protein synthesis, translocation, *de novo* folding, and higher ordered assembly of multiprotein complexes (Hendrick & Hartl 1993, Neuer *et al.* 2000). Among this multitude of functions, the ability of chaperones to mediate the assembly of oligomeric complexes is of particular interest as advances in functional proteomics have revealed that a significant portion of a cell's proteome realize their functional potential in multiprotein complexes rather than as singular entities (Sali *et al.* 2003).

This phenomenon is proving particularly true of the male gametes, which must complete several complex phases maturation in order to gain the functional competence to engage in one of the most intricate of

all cellular interactions, fertilization. As transcriptionally inactive cells, spermatozoa rely heavily upon post-translational modifications and remodeling of their constituent proteins to attain a state of functional maturity. For a sperm cell to interact with the oocyte, the activation of nascent, receptor-like proteins on the sperm surface is required, a process that has frequently been linked to the coordinated action of a subset of molecular chaperones (Ikawa *et al.* 2001, Asquith *et al.* 2004). Members of the HSP families, in addition to several other germ cell-specific chaperones, have been identified in both the mature spermatozoon of a number of species, including mouse (Asquith *et al.* 2004), human (Mitchell *et al.* 2007, Naaby-Hansen & Herr 2010), and pig (Spinaci *et al.* 2005), and in mature murine (Calvert *et al.* 2003) and bovine (Kawarsky & King 2001) oocytes and have been extensively implicated in gamete adhesion and fusion processes (Cayli *et al.* 2003, Asquith *et al.* 2004, Nixon *et al.* 2005, Huszar *et al.* 2007, Ikawa *et al.* 2011).

Preparation for oocyte interactions initially occurs through the formation of zona pellucida (ZP) binding sites during spermatogenesis (Huszar *et al.* 2007). Several molecular chaperones have been implicated in the remodeling of sperm membranes and the formation of oocyte binding domains during the production of

morphologically mature cells in the testes (Ikawa *et al.* 1997, Huszar *et al.* 2000). Though this prepares the spermatozoon for fertilization, additional phases of post-testicular maturation must be completed to attain a state of functional maturity. These events are initiated during their transit of the epididymis, wherein spermatozoa are bathed in a dynamic luminal micro-environment (Robaire *et al.* 2006, Cornwall 2009). Within this milieu, the sperm plasma membrane experiences the loss, gain, and modification of multiple proteins, a process that results in the attainment of the potential to move progressively and engage in oocyte interactions (Hermo *et al.* 1991). As discussed in the following sections, emerging evidence has implicated several HSP family chaperones in these modifications of the sperm plasma membrane during epididymal maturation (Lachance *et al.* 2010, Naaby-Hansen *et al.* 2010).

As a culmination of both testicular and post-testicular maturation, spermatozoa are able to reach a capacitated state as they progress through the female reproductive tract en route to the site of fertilization. As they ascend the female reproductive tract, the composition of sperm membrane proteins and lipids is subject to further dramatic alterations (Yanagimachi 1994, Harrison *et al.* 1996, Gadella & Harrison 2000, Gadella *et al.* 2008), resulting in the acquisition of the ability to recognize and bind to the outer vestments of the oocyte, the ZP. It is widely held that these binding events trigger acrosomal exocytosis and prime the cell for intercellular fusion with the oocyte. These collective events appear to be underpinned by a number of sperm surface protein complexes that comprise molecular chaperones, co-chaperones, and recognition proteins implicated in the specific interactions with oocyte ligands. Recently, a subset of these complexes has been demonstrated to undergo capacitation-associated assembly or activation (Asquith *et al.* 2005, Nixon *et al.* 2005). These chaperone-laden complexes may provide the basis for species-specific adhesion of spermatozoa to homologous oocytes and thus have been the major focus of studies of molecular chaperones in this field. In the ensuing review, we seek to discuss the role of molecular chaperones in the mediation of sperm–oocyte adhesion and fusion, two germ cell-specific events that are coordinated largely through the formation of multiprotein complexes.

The role of chaperones in the acquisition of ZP binding potential

Although the expression of chaperones is traditionally viewed as being induced by heat shock and other stresses, members of the HSP60, HSP70, and HSP90 families have proven to be abundant components of the sperm surface that are constitutively expressed in the male germline and function as critical mediators of protein maturation during spermatogenesis (Miller *et al.* 1992, Boulanger

et al. 1995). The generation of a mature spermatozoon requires multiple maturation steps; the first of these occurs in the testis and is assisted by the regulatory action of a diverse family of chaperones, including chaperonin-containing T-complex/TCP1-ring complex (CCT/TRiC), HSP60 (or HSPD1; Meinhardt *et al.* 1995), clusterin (Onoda & Djakiew 1990), HSPA2 (Huszar *et al.* 2000), and the testis-specific chaperones calmeglin (CLGN; Ikawa *et al.* 1997, Ohsako *et al.* 1994), and calsperin (CALR3; Ikawa *et al.* 2011), which are developmentally regulated and expressed in spermatogenic cells (see also Dun *et al.* (2012a)).

The role of these chaperones extends to arguably the most essential step in the acquisition of sperm fertilizing potential during spermatogenesis, the remodeling of the sperm morphology through the extrusion of excess cytoplasm, and the formation of a number of highly specialized domains. This remodeling phase culminates in the formation of both hyaluronic acid and ZP binding sites on the sperm plasma membrane that are fundamental to the processes of cumulus oophorus penetration and ZP interaction (Huszar *et al.* 2000, 2007). Little is known regarding the mechanisms that underpin ZP binding site formation; however, in immature human sperm, the presence of a low number of zona binding sites and hyaluronic acid receptor sites as well as a corresponding reduction in fertility potential has been causally related to the reduced expression of the 70 kDa testis-expressed chaperone, HSPA2 (Huszar *et al.* 2000, Celik-Ozenci *et al.* 2003).

In the human, HSPA2 is expressed in the synaptonemal complex of spermatocytes and is again synthesized during late spermiogenesis concurrently with cytoplasmic extrusion and sperm plasma membrane remodeling, processes that require major sperm protein translocations (Huszar *et al.* 2000). In concert with a number of other testis-expressed chaperones (see below), HSPA2 assists in the mediation of correct protein folding and translocation to appropriate subcellular domains to produce a morphologically mature spermatozoon (Huszar *et al.* 2000, 2003). The importance of HSPA2 in spermatogenic differentiation is reinforced by studies that indicate that the diminished expression of this chaperone is associated with apoptosis (Dix *et al.* 1996, Nasr-Esfahani *et al.* 2010), DNA fragmentation (Kovanci *et al.* 2001), a lack of histone–protamine substitution (Nasr-Esfahani *et al.* 2001), increased levels of lipid peroxidation (Huszar *et al.* 2000), increased frequency of chromosomal aneuploidies (Nasr-Esfahani *et al.* 2001), as well as IVF failure (Huszar *et al.* 1992, 2003). Indeed, in the mouse, the targeted elimination of HSPA2 leads to a complete arrest of spermatogenesis (Allen *et al.* 1996, Mori *et al.* 1997). These defects appear to be a result of misfolding, misexpression, or incorrect processing of proteins during spermatogenesis (Neuer *et al.* 2000).

In some instances, chaperones, such as the testis-specific calmegin, remain imperative to sperm function despite the fact that they are not expressed in the mature cell. Such chaperones are necessary as they ensure correct folding of endoplasmic reticulum glycoproteins that are destined to play a more direct role in zona adhesion in addition to promoting their delivery to the appropriate plasma membrane domain (Ikawa *et al.* 1997). Indeed, disruption of the calmegin (Ikawa *et al.* 1997, Ikawa *et al.* 2001) and calsperin (Ikawa *et al.* 2011) genes in mice has been demonstrated to compromise male fertility due to the impaired formation of testis-specific t-fertilin (ADAM1A and ADAM2, where ADAM denotes a disintegrin and metalloprotease) and sperm surface s-fertilin (ADAM1B and ADAM2) complexes. Such defects are causally associated with impaired zona binding and reduced sperm transport through the uterotubal junction of the female reproductive tract (Ikawa *et al.* 2001). Interestingly, this phenotype is shared among several knockout models, including calmegin, calsperin, ADAM1A, and ADAM2, and appears to be attributed to the absence of ADAM3 from spermatozoa in each instance (Yamaguchi *et al.* 2006). Such a finding suggests that the assembly of the fertilin complexes by chaperones during spermatogenesis is essential for the formation of ZP recognition domains as well as the processing and/or escort of key receptor proteins such as ADAM3 (Muro & Okabe 2011).

Following their release from the testes, the second major phase of sperm maturation occurs as the cells progress through the dynamic environment of the epididymis, the extratesticular duct that connects the testis and the vas deferens. As a culmination of epididymal maturation, spermatozoa acquire the potential to move progressively and interact with the ZP. Chaperone proteins including clusterin (Hermo *et al.* 1991), HSPA5 (Lachance *et al.* 2010, Naaby-Hansen *et al.* 2010), and HSPD1 (Asquith *et al.* 2005) are thought to regulate epididymal maturation through their indirect roles in promoting the remodeling of sperm surface architecture (Dun *et al.* 2012a). Although the mechanisms by which proteins are incorporated into spermatozoa during epididymal maturation are yet to be completely resolved, it is postulated that this process occurs through sperm membrane fusion with small, epididymal exosomal vesicles termed epididymosomes (Frenette & Sullivan 2001, Sullivan *et al.* 2007) and/or amorphous, electron-dense structures termed dense bodies (Asquith *et al.* 2005). The expression of several molecular chaperones in epididymosomes (Frenette & Sullivan 2001, Saez *et al.* 2003, Sullivan *et al.* 2005) and dense bodies, including HSPD1, suggests that these proteins may facilitate the bulk transfer of new protein onto the sperm surface during epididymal maturation (Asquith *et al.* 2005, Dun *et al.* 2011). In addition, there is some evidence to suggest a correlation between the

presence of clusterin-positive sperm and bull fertility (Ibrahim *et al.* 2000).

In mature mouse sperm, HSPD1 has been shown to subsequently interact with additional chaperones HSPÉ1 and CCT/TRiC as well as a putative ZP receptor candidate ADAMTS10 (Walsh *et al.* 2008, Dun *et al.* 2011, 2012b), which exemplifies the important role this chaperone may play in epididymal sperm surface remodeling to prepare the cell for ZP interactions. In support of this model, HSPA5 has also been identified in epididymosomes (Lachance *et al.* 2010) and together with its putative client protein, ADAM7, is also transferred to the sperm surface during epididymal maturation (Oh *et al.* 2009). Within spermatozoa, these proteins form a multimeric complex (comprising an additional chaperone, calnexin, and integral membrane protein 2B), which has been implicated in sperm–oocyte interactions (Han *et al.* 2011). These findings draw interesting analogies with a number of other cell types, including that of metastatic cancers, where chaperones have been intimately tied to the function of metalloproteases, raising the possibility that this form of interaction may regulate a range of biological and pathological processes (Ikawa *et al.* 2010).

Despite the collective importance of these maturation stages in the acquisition of ZP binding potential, they do not represent the final changes necessary for the spermatozoon to engage in fertilization. Rather, a further pivotal maturation phase must take place post-ejaculation within the female reproductive tract.

Multiprotein complexes in sperm capacitation and ZP interaction

Upon reaching the site of fertilization, the ampulla, spermatozoa must penetrate two barriers before fusing with the oocyte plasma membrane, or oolemma. The first of these is a hyaluronic acid-rich stratum of cumulus cells that surround the oocyte and the second is the extracellular matrix of the oocyte itself, the ZP (Hartmann *et al.* 1972). Despite the development of both ZP and hyaluronic acid binding sites during spermatogenesis and the acquisition of binding potential as spermatozoa transit the epididymis, these cells require a distinct period of residence within the female reproductive tract before they are able to successfully partake in such interactions (Austin 1951, Chang 1951). The collective changes that spermatozoa undergo within this environment, termed capacitation, enable the cells to respond to signals arising from the cumulus oocyte complex and complete a process of acrosomal exocytosis, rendering them competent for fusion with the oolemma.

The ZP is comprised of a suite of sulfoglycoproteins, namely ZP1, ZP2 and ZP3, that are highly conserved in most mammalian species (though an additional ligand, ZP4/B, has been reported in human and pig oocytes)

(Wassarman *et al.* 1999, Lefievre *et al.* 2002, Yonezawa *et al.* 2012). It is generally held that these ligands govern sperm binding in most species (see also Reid *et al.* (2011)). Remarkably, however, various models are currently still under consideration regarding the identity of the primary sperm receptor within the ZP and the mechanisms by which spermatozoa adhere to this matrix (see also Visconti & Florman (2010)). Similarly, investigations into the identity of the corresponding sperm surface receptor(s) that recognize the appropriate ligand(s) on the ZP have also failed to provide definitive answers. Indeed, there is a growing literature of murine knockouts of auspicious receptor protein candidates (including β -1,4-galactosyltransferase (GALT1), arylsulphatase A (ARSA), and sperm adhesion molecule 1 (SPAM1); for a complete list, see Ikawa *et al.* (2010)) that each fail to result in complete infertility (Hess *et al.* 1996, Asano *et al.* 1997, Baba *et al.* 2002). Rather, various degrees of reduced binding capability are exhibited, raising the possibility that this process encompasses a degree of functional redundancy and that a number of sperm proteins act in concert to mediate ZP adhesion. The coordination of the activity of these proteins to ensure productive ZP interactions is thus emerging as an important research focus.

In most eutherian mammals, sperm capacitation is thought to be initiated by the activation of a cAMP-mediated pathway that culminates in the tyrosine phosphorylation of multiple sperm proteins (Visconti *et al.* 1995a, 1995b, Leclerc *et al.* 1996). Molecular chaperones feature prominently among this suite of proteins, with HSP90AA1, HSP90B1 and HSPD1 being among the proteins revealed to display tyrosine phosphorylation as a consequence of capacitation (Ecroyd *et al.* 2003, Asquith *et al.* 2004). Current models suggest that the phosphorylation of these chaperones during capacitation triggers their active role in the assembly of ZP recognition proteins into complexes and/or the translocation of these complexes to the surface of spermatozoa in preparation for fertilization (Ecroyd *et al.* 2003, Asquith *et al.* 2004, Nixon *et al.* 2005, Gadella 2008). Further to this indirect role in gamete adhesion, sperm surface chaperones also have purported functions as adhesion molecules that mediate the recognition of sulfoglycolipids during gamete binding (Boulanger *et al.* 1995, Mamelak & Lingwood 2001).

Recently, the technique of blue native PAGE (BN-PAGE), which was originally developed for the analysis of electron transport chain multienzyme complexes (Schägger & von Jagow 1991, Schägger *et al.* 1994), has been adapted for the assessment of multimeric sperm surface complexes in mice and humans (Dun *et al.* 2011, Redgrove *et al.* 2011). This technique allows for the electrophoretic resolution of native protein complexes that retain their biological activity. In human and mouse spermatozoa, the use of BN-PAGE in parallel with Far-Western blotting with whole solubilized zonae has

revealed several primary multiprotein complexes that possess affinity for homologous ZP (Dun *et al.* 2011, Redgrove *et al.* 2011).

One such complex has been reported to comprise the protein components of the CCT/TRiC complex (CCT1–CCT8), a double-ring structure that functions as a molecular chaperone with a key role in regulating the formation of multiprotein complexes (Feldman *et al.* 1999, Guenther *et al.* 2002). Putative evidence in the form of co-immunoprecipitation, co-localization, and proximity ligation assays has identified ZP binding protein 2 (ZPBP2) as one of the most compelling client proteins for the CCT/TRiC complex in mature spermatozoa (Dun *et al.* 2011, Redgrove *et al.* 2011). Originally implicated in secondary ZP binding, a more recent study has shown that male mice null for ZPBP2 are subfertile and display defects in ZP interaction and penetration (Lin *et al.* 2007). In mice, there is additional evidence that certain CCT/TRiC complex subunits are translocated to the sperm surface during sperm capacitation (Dun *et al.* 2011).

Another prominent class of chaperones that has been identified on the sperm surface and implicated in the regulation of ZP interactions is the HSP70 family (Naaby-Hansen *et al.* 2010). As with the CCT/TRiC complex, HSP70 family chaperones also have well-documented roles in the facilitation of both transmembrane protein transport and assembly of stable protein complexes (Mayer & Bukau 2005). One member of the HSP70 family that displays exclusive (mouse) or predominant (human) expression in the testes appears to be essential for male fertility. Indeed, aberrant expression of this chaperone, HSPA2, has been correlated with a phenotype of severe male factor infertility in humans, specifically affecting the ability of spermatozoa to interact with homologous oocytes *in vitro* (Eddy 1999, Huszar *et al.* 2007). In both mice and humans, HSPA2 has a fundamental role in spermatogenesis, with targeted deletion of the protein in the former species leading to an early arrest of this process and a concomitant absence of spermatozoa (Eddy 1999). In humans, the expression levels of HSPA2 have been positively correlated with the success of fertilization *in vitro* (Huszar *et al.* 2000, 2006, Cayli *et al.* 2003) and hence are purportedly able to predict the fertility status of men with a high degree of accuracy (Ergur *et al.* 2002).

Characterization of HSPA2 in our own laboratory has revealed that this chaperone is present in the acrosomal domain of human spermatozoa and is a component of at least five high-molecular-mass protein complexes (Redgrove *et al.* 2012), including a subset of those shown previously to possess ZP affinity (Redgrove *et al.* 2011). Consistent with these data, we have secured evidence that the most dominant of the HSPA2 complexes contains two additional proteins, both of which have been previously implicated in sperm–zona interactions (Redgrove *et al.* 2012). Furthermore, in agreement with

the published results of Huszar *et al.*, we have been able to demonstrate a significant reduction in HSPA2 levels in the spermatozoa of men with isolated lesions in their ability to engage in interactions with ZP of homologous oocytes *in vitro* (Redgrove *et al.* 2012). Our current work is focusing on whether the deficit in ZP adhesion either results from aberrant formation of ZP binding sites in the early stages of spermiogenesis (Huszar *et al.* 2000) or may be the result of the inability of HSPA2 to participate in sperm surface remodeling events during capacitation such as facilitating the assembly and/or presentation of ZP receptors on the sperm surface in preparation for ZP interaction.

In addition to our own work on the assembly of sperm surface complexes, Han *et al.* have independently identified an alternative chaperone-laden multiprotein complex on the surface of mouse spermatozoa. Interestingly, as documented above, this complex, comprising HSPA5, calnexin, integral membrane protein 2B, and ADAM7, is apparently assembled during capacitation (Han *et al.* 2011). While the function of this complex has yet to be fully elucidated, the expression of ADAM7 has been linked to the presence of additional ADAM proteins, ADAM2 and ADAM3 (Kim *et al.* 2006), that are important for adhesion of spermatozoa to the ZP (Muro & Okabe 2011). In addition, it is known that HSPA5 is involved in promoting adhesion of high-quality spermatozoa to oviductal epithelial cells (OEC) in the isthmus of the female reproductive tract. The formation of this reservoir is believed to have pro-survival effects in terms of maintaining sperm in a non-capacitated, quiescent state in preparation for the oocyte to be released to the ampulla (Topfer-Petersen *et al.* 2002). Interestingly, the chaperones HSPD1 and HSPA5 have also been localized to the surface of bovine OEC and have thus been implicated in sperm–OEC binding (Boilard *et al.* 2004).

Also consistent with our own work, the complex identified by Han *et al.* was shown to reside in membrane microdomains or lipid rafts, specialized regions of the membrane that provide a platform for the functional assembly and presentation of multiprotein complexes (Stein *et al.* 2006, Nixon *et al.* 2009, Han *et al.* 2011). The partitioning of chaperone complexes into the raft environment has also been observed for HSPA2 in human spermatozoa (Nixon *et al.* 2011) and for components of the CCT/TRiC complex in mouse spermatozoa (Dun *et al.* 2011). These membrane domains also comprise a number of additional putative ZP receptor proteins, including GALT1, ZP3R, and SPAM1, reinforcing their role in the remodeling of the sperm surface and in ZP binding (Fig. 1; Nixon *et al.* 2009, Asano *et al.* 2010). The mechanism(s) by which such proteins are recruited to the lipid rafts are yet to be resolved; however, HSPA2 has been reported to bind via its ATPase domain to 3'-sulfolactosylglycerolipid, the

major glycoprotein identified within sperm lipid rafts (Mamelak & Lingwood 2001).

In addition to the putative role of lipid rafts in repositioning of key chaperone complexes and ZP receptor proteins, there is also compelling evidence that many putative ZP receptors, such as ARSA and ZP3R, as well as several molecular chaperones show a capacitation-dependent relocation from intracellular sites such as the acrosome to the sperm surface to prime the cells in advance of their interactions with the ZP (Nixon *et al.* 2009). It has been proposed that intimate contact between the outer acrosomal membrane and the sperm plasma membrane is mediated through the binding of complementary soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, leading to the formation of fusion pores that provide a route for the migration of enzymes to the sperm surface before the complete loss of the acrosomal contents (Søgaard *et al.* 1994, Blas *et al.* 2005, Tsai *et al.* 2007). In support of this model, a study by Brahmaraju *et al.* (2004) demonstrated that administration of antibodies to VAMP and SNAP into the acrosomal vesicle inhibited sperm–ZP binding in the mouse.

This progressive priming of the sperm surface has raised questions regarding the all or none nature of acrosomal exocytosis. Nevertheless, the functional assembly of SNARE complexes also appears to underpin the prolonged sperm membrane fusion events that permit a complete loss of the acrosomal contents (Tsai *et al.* 2010). Though it is widely held that contact with the ZP initiates this acrosomal exocytosis in most mammalian species, a number of studies performed in the mouse have demonstrated that spermatozoa that begin acrosomal exocytosis before contact with the ZP are still able to fertilize the oocyte (Nakanishi *et al.* 1999, Jin *et al.* 2011). This phenomenon may also be true of guinea pig (Huang *et al.* 1981) and hamster spermatozoa (Yanagimachi & Phillips 1984). Such findings suggest an important role for the cumulus oophorus in the initiation of the acrosome reaction and raise concerns regarding the ability of *in vitro* studies performed with cumulus-denuded oocyte zona structures to accurately report on the true nature of the acrosome reaction and indeed ZP interaction.

Notwithstanding such controversy, chaperone-like molecules have also been implicated in acrosomal exocytosis by virtue of their ability to promote the assembly of glutamine-containing SNAREs (Q-SNAREs) and arginine-containing SNAREs (T-SNAREs) into tight ternary complexes (Tomes *et al.* 2002, Sørensen 2005). Interestingly, elegant studies in pigs have demonstrated that capacitation induces stable docking of the sperm plasma membrane with the outer acrosomal membrane in preparation for fertilization (Tsai *et al.* 2010). More recent studies performed by Tsai *et al.* (2012) have also provided evidence for the presence of unilamellar mixed vesicles that, among other important functions, allow the

Figure 1 The mammalian oocyte and its complementary receptors and chaperones on the sperm surface. (A) The balance of evidence suggests that the mammalian oocyte selectively interacts with the spermatozoon through glycans conjugated to ZP3 and/or elements of the ZP2 and ZP3 peptide backbones. (B) While the identity of the complementary sperm receptors has yet to be fully elucidated, it is known that spermatozoa possess membrane rafts that undergo a capacitation-dependent reorganization to become confined within the apical region of the sperm head. These membrane domains have been shown to contain a number of putative ZP receptor proteins (orange), including SPAM1, ARSA, GALT1, and ZP3R, as well as several molecular chaperones (blue) such as CCT/TRiC, HSPA2, HSPD1, and HSPE1. On the basis of this evidence, it has been proposed that membrane rafts serve as a platform for the chaperone-mediated assembly of functional oocyte–receptor complexes on the sperm surface. Among these putative complexes that have been identified to date are those that comprise CCT/TRiC/ZPBP2, HSPD1/HSPE1/ADAMTS10, HSPA2/ARSA/SPAM1, and HSPA5/ADAM7/calnexin. Though we have much to learn regarding the specific interactions between sperm surface receptors, chaperones, and ZP ligands, some examples of proposed interactions include ARSA interaction with sulfated ZP2/ZP3 glycans via a positively charged active site pocket; GALT1, a carbohydrate binding protein that specifically recognizes terminal *N*-acetylglucosamine (GlcNAc) sugar residues on ZP3; and ZP3R, a member of the superfamily of C3/C4 binding proteins that interacts with terminal α -galactose (α -Gal) residues on ZP3. Such a large number of putative sperm–ZP receptors suggest that a high level of functional redundancy is associated with this critical aspect of the fertilization cascade.

recruitment of secondary ZP binding proteins at the sperm surface and possess a novel trimeric SNARE complex consisting of syntaxin 3, SNAP23, VAMP2, and an additional protein, complexin 2. The energy released by the formation of such complexes is in turn used to initiate membrane fusion by pulling together the plasma membrane and inner acrosomal membrane of the spermatozoon (Tomes *et al.* 2002). The completion of this process is critical in the exposure of sperm domains that participate in the downstream events of fertilization: oolemma binding and fusion.

Molecular chaperones in gamete fusion

Sperm–oocyte fusion occurs between the inner acrosomal membrane of the sperm cell and the microvillar-rich region of the oocyte plasma membrane (Johnson *et al.* 1975). Specific fusion proteins tightly regulate these events through interactions with lipids and other protein ligands. Through comprehensively studied membrane fusion processes such as vesicle trafficking and virus–cell fusion, it has been revealed that membrane fusion often involves an initial tethering step, followed by the

activation of the fusion machinery, the close apposition of the lipid bilayers, and then the final fusion of the membranes (Jahn & Grubmüller 2002).

Although evidence for the role of chaperones in the mediation of intercellular gamete fusion is limited, given the mounting support for the involvement of chaperone-laden complexes in the regulation of ZP binding, it is expected that this ubiquitous class of proteins may also assist in the activation of fusion machinery and/or organization of functional fusion complexes. Analogous to the regulation of sperm–ZP interaction, a functional redundancy exists between the numerous sperm proteins required for efficient fusion of the sperm cell to the oolemma. One sperm protein that has been established as essential for sperm–oocyte fusion is IZUMO1. IZUMO1 was originally identified in spermatozoa through the use of anti-sperm monoclonal antibodies that were shown to block IVF and, upon being knocked out, *Izumo1*-null sperm were shown to be unable to fuse with the oolemma (Inoue *et al.* 2005).

Notwithstanding recent evidence from knockout models, an extensive body of literature suggests that ADAMs are also vital for sperm–egg adhesion and fusion (Primakoff *et al.* 1987, Cho *et al.* 1998, Nishimura *et al.* 2001, Choi *et al.* 2003). ADAMs share adhesion-mediating motifs that allow them to interact with integrins on the egg surface. However, this interaction occurs in a coordinated manner, possibly with IZUMO1 and the fusogen, angiotensin-converting enzyme 3 (ACE3; Krege *et al.* 1995), and as such no ADAM has thus far been found to be singularly essential for sperm–egg interaction. Preliminary evidence of the interaction between IZUMO1, ACE3, and the ADAM family of proteins was afforded by Ellerman *et al.* (2009) who indicated that ACE3 and ADAM proteins possess an Izumo domain and are able to form heteromultimeric complexes on spermatozoa. Given that ADAMs are able to form heteromultimeric complexes and have been shown to interact with several molecular chaperones during the maturation events upstream of fusion, it is tempting to speculate that molecular chaperones may coordinate the actions of ADAMs during fusion; however, such a role is yet to be investigated.

In terms of the maternal contributions to membrane fusion, egg plasma membrane tetraspanins and integrin receptors are the key molecules to have been implicated in sperm adhesion and fusion processes. Of particular importance are CD9 and CD81, members of the tetraspanin family that are present on the oolemma and essential for sperm–egg fusion (Kaji *et al.* 2000, Le Naour *et al.* 2000, Miyado *et al.* 2000). Remarkably, CD9 and CD81 appear to work in concert to facilitate fusion and when knocked out simultaneously, *Cd9*^{−/−}/*Cd81*^{−/−} females are completely infertile (Rubinstein *et al.* 2006). Though the precise role of these tetraspanins is unclear (for a current review, see Evans (2012)), CD9 has interestingly been reported to contribute to fusion

through the organization of functional oolemmal multi-meric complexes (Chen *et al.* 1999).

Although a link between oocyte tetraspanins and the action of molecular chaperones has not been developed, tetraspanins have been shown to interact with chaperones in other cell types. Working in a B-cell model, it has been shown that the chaperone, calnexin, and the tetraspanins, CD9 (Rubinstein *et al.* 1997) and CD82 (Cannon & Cresswell 2001), can be co-immunoprecipitated from this cell type. Additionally, the endoplasmic reticulum transmembrane domain chaperone BAP31 has been identified as a constituent of a high-molecular-weight complex in which it associates with the integrin CD11c/CD18, regulating the mobilization of the integrin complex to the neutrophil cell surface from secondary granules (Zen *et al.* 2009). This activity may be mediated through the chaperones' association with hydrophilic residues of the transmembrane domains of its client proteins (Adachi *et al.* 1996). In turn, BAP31 has also been shown to form complexes with calnexin (Zuppin *et al.* 2002) and is thought to regulate tetraspanin trafficking through the assembly of tetraspanin–calnexin complexes (Berditchevski & Odintsova 2007).

The presence of multiprotein complexes on the surface of the oocyte has not been extensively investigated due to the difficulty in acquiring significant biological material to characterize the proteome or perform analyses such as BN-PAGE. Despite this, the individual chaperone proteins, calreticulin, calnexin, HSPA1A, HSPA5, HSP90AA1, and HSP90B1, have been confirmed on the surface of mature mouse oocytes (Calvert *et al.* 2003) and may act in a coordinated manner to activate fusion machinery on the surface of the oocyte. In an independent study, calreticulin has also been found to localize to the cortical granules of hamster oocytes and appears to be released into the perivitelline space following oocyte activation (Munoz-Gotera *et al.* 2001), raising the possibility that it is involved in the priming of oocytes in preparation for fusion with a spermatozoon.

Of the chaperone proteins identified on the oolemma, HSP70 is constitutively synthesized in the preovulatory mouse oocyte (Calvert *et al.* 2003) as well as the mature bovine oocyte (Kawarsky & King 2001). Following zygotic gene activation in the two-cell mouse embryo, HSP70 is one of the primary genes to be expressed. From this observation, it is anticipated that chaperones of the HSP family that are found in the mature egg may be required to ensure accurate translation and folding of nascent proteins during activation of the zygotic genome. Furthermore, HSP70 and an additional chaperone protein, HSPA5, possess anti-apoptotic activity that may imply that HSPs have uncharacterized functions in terms of promoting cell survival (Heikkila *et al.* 1986).

Figure 2 Chaperone expression during capacitation, ZP interaction, and sperm–oolemma fusion. A large suite of molecular chaperones have been implicated in the structural and functional maturation of mammalian spermatozoa within the testes and male reproductive tract. The presence of a number of these chaperones in ejaculated spermatozoa raises the possibility that they fulfill important functions during the capacitation of these cells and in facilitating their interaction with the oocyte within the ampulla of the female reproductive tract. Consistent with this notion, molecular chaperones including CCT/TRiC, HSPA2, HSPA5, HSPD1, HSP90AA1, and HSP90B1 have been shown to be targets for capacitation-associated modification and/or have been indirectly implicated in zona recognition. Specifically, chaperones such as these appear to assist in priming the sperm surface architecture for ZP interactions by virtue of their ability to assemble/present and/or activate ZP–receptor complexes. Although the role of chaperones in downstream interactions such as adhesion and fusion to the oolemma is less certain, a number of them including calreticulin, HSPA1A, HSPA5, HSP90AA1, and HSP90B1 have been localized to the surface of the oolemma and are thus well positioned to participate in these processes.

Conclusions

Though it is widely accepted that molecular chaperones are vital for the maturation of mammalian spermatozoa in the testis and epididymis, techniques such as BN-PAGE have led to the discovery of functional, chaperone-laden complexes in the mature sperm cell. In addition, murine gene manipulation studies and bioassays have begun to elucidate the functional roles of these chaperones in gamete interactions. Such studies have established a link between the phosphorylation of molecular chaperones during sperm capacitation and the formation and/or presentation of multiprotein complexes on the surface of the cell. Many of these complexes comprise putative ZP receptor proteins, and accordingly, they have been shown to possess affinity for homologous zonae. The presence of multiple chaperone-laden, ZP recognition complexes on the surface of the spermatozoon at the time of fertilization is consistent with the high level of functional redundancy that characterizes this intricate adhesion event.

Downstream of ZP interactions, little is known regarding the role of chaperones in the activation of fusion machinery and the subsequent adhesion and fusion of a spermatozoon to the oolemma. It is of interest, however, that several chaperone proteins have been identified on the surface of the oocyte. This discovery raises the possibility that chaperones mediate the formation of multiprotein complexes on the female gamete that may assist in fusion and could represent a maternal selection of essential protein folding machinery. Given the similar number of gamete fusion candidates to those involved in sperm–ZP binding, it is highly likely that multiple proteins regulate this process through the formation of functional complexes. Whether molecular chaperones are essential for the coordination of such complexes is yet to be determined and is a focus for future research into the elucidation of gamete interactions in our laboratory.

In summary, the key roles attributed to chaperone expression during capacitation, ZP interaction, and

sperm–oolemma fusion (Fig. 2) highlight the importance of the chaperone machinery in gamete biology and may set the stage for an exciting new facet of proteomic research.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported in this review.

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