The role of molecular chaperones in spermatogenesis and the post-testicular maturation of mammalian spermatozoa

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TABLE OF CONTENTS

- Introduction
- Methods
- Results: families of molecular chaperones implicated in spermatogenesis and/or the post-testicular maturation of mammalian spermatozoa
  - Chaperonin containing T-complex/TCPI-ring complex
  - Heat shock protein 60
  - Heat shock protein 70 family
  - Calnexin/Calsperin
  - Calreticulin
  - Heat shock protein 90
  - Protein disulphide isomerases
- Conclusion

BACKGROUND: Spermatogenesis culminates in production of one of the most highly differentiated cells in biology, the spermatozoon. The gametes that emerge from the testes are, however, functionally immature and only acquire full functionality once they have completed a process of post-testicular maturation in the epididymis and female reproductive tract. Remarkably, this acquisition of sperm function occurs while these cells are transcriptionally and translationally silent and is therefore highly dependent on post-translational modifications to their existing protein complement. In this review, we consider the emerging roles of several prominent molecular chaperone families in orchestrating both the morphological transformation of male germ cells during spermatogenesis and their functional transformation during sperm maturation.

METHODS: Journal databases were searched using key words, including chaperone, heat shock protein, testes, spermatogenesis, spermatozoa, epididymal maturation, capacitation and fertilization.

RESULTS: In the past two decades, molecular chaperones have been acknowledged to play key roles in controlling both the morphological transformation of germ cells during spermatogenesis and the post-testicular maturation of these cells as they transit the male and female reproductive tracts. Furthermore, there is mounting evidence that aberrant chaperone expression may be a major contributing factor to the defective sperm function seen in many cases of male infertility.
**CONCLUSIONS:** Molecular chaperones are critically involved in all phases of sperm development. Targeted disruption of these proteins has the ability to arrest spermatogenesis, compromise sperm maturation and inhibit fertilization. These proteins therefore hold considerable promise as targets for novel contraceptive strategies and as diagnostic biomarkers for male infertility.

**Key words:** spermatozoa / molecular chaperone / sperm maturation / fertilization

## Introduction

Spermatogenesis involves the passage of diploid germ cells through the reductive divisions of meiosis in order to generate round haploid spermatids that metamorphose into one of the most specialized cells in biology, the spermatozoon, in a process known as spermiogenesis. Remarkably, the morphological transformations associated with spermiogenesis occur in the complete absence of gene transcription, beautifully illustrating how regulated protein translation can control the phenotypic fate of cells during development. The end result of this process is a unique, highly polarized cell that is designed to deliver the paternal genome, centriole and possibly, key mRNA species to the oocyte at the time of fertilization (Hermo et al., 2010a, b).

Notwithstanding the morphological specialization generated during spermatogenesis, spermatozoa are released from the testes in a functionally immature state. The functional transformation of these cells occurs as they transit the epididymis and ascend the female reproductive tract. Taking place in the virtual absence of gene transcription and de novo protein synthesis (Kierszenbaum and Tres, 1975; Meistrich et al., 1978; Balhorn et al., 1984; Heidaran et al., 1988). Sperm maturation therefore depends upon the loss, modification and/or remodelling of existing sperm proteins in response to cues delivered by the male and female reproductive tracts. While many aspects of spermatogenesis and post-testicular sperm maturation remain to be fully elucidated, it is becoming increasingly apparent that these elaborate processes are carefully regulated by a myriad of gene products that are expressed in a phase-specific manner (Hermo et al., 2010a, b). Through the application of advanced biochemical, molecular and proteomic technologies, many of these products have been identified. Interestingly, several classes of molecular chaperones are among those that have proved to play indispensable roles during all the stages of sperm development.

Molecular chaperones are a large family of structurally diverse proteins that are expressed virtually in all cell types (Ellis, 1987, 1996). More than 20 chaperone families, differing primarily with respect to their molecular weight and structural characteristics, have now been described. Owing to the fact that they were originally identified on the basis of their ability to confer cellular resistance to environmental stressors, the majority of these chaperone families are referred to as cell stress response or, more commonly, heat shock proteins (HSPs) (Ritossa, 1996). In mammals, the HSPs are commonly divided into the HSP100 (HSPH), HSP90 (HSPC), HSP70 (HSPA), HSP60 (HSPD), HSP40 and HSP27 (HSP8) families (Buchner, 1996; Bukau and Horwich, 1998; Narberhaus, 2002). The human genome contains several members from each gene family, a redundancy that may relate to differences in intra-organelle compartmentalization in addition to tissue or development-specific expression patterns (Vos et al., 2008). However, the overlapping expression of many different molecular chaperone families or family members indicates that their specialized functions are of utmost importance (Vos et al., 2008). Indeed, there is compelling evidence that in addition to their archetypal protective roles, molecular chaperones also participate in a number of normal cellular functions, including metabolism, growth, differentiation and apoptosis (Bukau et al., 2006). Molecular chaperones fulfill these functions by virtue of their ability to selectively recognize and interact with hydrophobic domains that are transiently exposed in their client proteins. Such interactions prevent inappropriate association or aggregation and direct the proteins into productive folding, transport or degradation pathways (Fig. 1). Although molecular chaperones were initially thought to be restricted to intracellular organelles, a growing body of evidence suggests that they do exist in other locations, including the cell surface, where it appears that they fulfill a number of novel secondary roles (Gupta et al., 2008; Triantafillou et al., 2008; Naaby-Hansen and Herr, 2010; Sims et al., 2011).

In the current review, we consider the established and rapidly emerging roles of several of the more prominent molecular chaperone families in the formation of a functional spermatozoon (Supplementary data, Table S1). Specifically, we have sought to highlight their roles in promoting the morphological differentiation of the male gametes during spermatogenesis and their subsequent post-testicular functional transformation in the epididymis and female reproductive tract. We present compelling evidence that molecular chaperones are critically important in cellular remodelling events and that aberrant expression is associated with arrested spermatogenesis and pronounced defects in sperm function. Further analysis of molecular chaperones is therefore warranted as a means of providing important insights into some of the most challenging questions concerning the molecular mechanisms regulating sperm function.

## Methods

Journal databases, including PubMed, Science Direct, Ovid, Wiley Online Library, Oxford and Google Scholar were searched using key words, including chaperone, heat shock protein, testis, spermatogenesis, spermatozoa, epididymal maturation, capacitation and fertilization. The most commonly used search included chaperone AND varying combinations of the above words. Journal articles were included based on their quality and relevance. Our journal database searches were not restricted by species; however, we focused our review on the human and mouse as these species were found to represent the two most widely studied models of chaperone function.
Results: families of molecular chaperones implicated in spermatogenesis and/or the post-testicular maturation of mammalian spermatozoa

Chaperonin containing T-complex/TCPI-ring complex

One of the most highly expressed molecular chaperone complexes in haploid germ cells is the chaperonin containing T-complex/TCPI-ring complex (CCT/TRiC) (Silver, 1985). CCT/TRiC is a large, double ring structure possessing a central cavity that is responsible for binding unfolded or denatured polypeptides (Fig. 2Ai). Each ring is composed of eight (CCT1–CCT8) unique subunits of between 52 and 65 kDa (Lewis et al., 1992; Hartl, 1996; Liou and Willison, 1997; Kubota et al., 1999) that each appear to be essential for its chaperoning activity (Silver et al., 1979; Miller et al., 2006). Substrate recognition takes place through diverse mechanisms involving hydrophobic and electrostatic interactions. Similar to other chaperone systems, CCT/TRiC also binds ATP and hydrolyses it during protein-folding cycles, with flexible protrusions located in the apical domain in each of the CCT subunit acting as a lid that is responsible for closing the central cavity (Gutsche et al., 1999; Leroux and Hartl, 2000; Meyer et al., 2003; Fig. 2Aii).

Despite the fact that the chaperonin activity of the CCT/TRiC complex has primarily been assessed on the basis of its involvement in the assembly of actin and tubulin filaments (Gao et al., 1992; Lewis et al., 1992; Marco et al., 1994; Siegers et al., 2003), it now appears that it may be far more promiscuous than previously appreciated. Indeed, the presence of eight different subunits enables the complex to potentially interact with a large number of structural features and motifs and thus accommodate a broad variety of substrates (Dunn et al., 2001; Gomez-Puertas et al., 2004). Consistent with this proposal, estimates now suggest that the CCT/TRiC complex may act as the folding machinery for as many as 5–10% of newly synthesized cytosolic proteins (Kubota et al., 1994; Thulasiraman et al., 1999; Yam et al., 2008). The fine-tuning of protein processing by the CCT/TRiC complex involves many co-chaperones that act upstream to facilitate substrate transfer to the central cavity of the complex and may also be responsible for the protection of the newly synthesized polypeptide chains to minimize their chances of aggregation and misfolding. It has been shown that the activity of the complex is also regulated by the phosphorylation of certain CCT subunits (Yam et al., 2008).

The original TCP-1 polypeptide was identified as a highly expressed mouse testicular protein (Silver et al., 1979; Silver, 1981) encoded by a gene located within the t-complex on chromosome 17 (Silver et al., 1980). The t-complex is of interest as it is known to harbour genes that influence mouse development and male fertility (Silver et al., 1979). For instance, it has been variously reported that t-specified differences exist in sperm antigenic properties (Bennett et al., 1975;
Silver, 1985), metabolic levels (Artzt et al., 1979; Cheng and Bennett, 1980) and sperm–zona pellucida (ZP) receptor activity (Ginsberg and Hillman, 1975). Within the testes, this complex is believed to be essential for the cytodifferentiation of spermatids (Soues et al., 2003). In addition to its putative role in spermatogenesis, several components of the CCT/TRiC complex have also been identified as secretory products within the epididymal fluid of the bull (Frenette et al., 2006; Belleannee et al., 2011). Indeed, proteomic analysis of bovine epididymal fluid has revealed the presence of three of the eight CCT/TRiC subunits (CCT2, CCT7 and CCT8) which, together with a subset of additional chaperones, account for as much as 6% of the total epididymal protein content (Belleannee et al., 2011). At present, it remains to be determined whether the additional CCT subunits are also present in the epididymal fluid, and therefore whether they exist in a functional complex within this extracellular environment. Nevertheless, the extracellular location of these chaperonin subunits is very intriguing, particularly as some have been shown to reside within prostasome-like (Ronquist and Brody, 1985; Fornes et al., 1995) structures known as epididymosomes (Yanagimachi et al., 1985; Fornes et al., 1991; Frenette and Sullivan, 2001). These membranous vesicles have been shown to interact with the sperm plasma membrane in vivo (Yanagimachi et al., 1985) and promote the transfer of cargo proteins, thereby contributing to the functional maturation of these cells (Legare et al., 1999; Saez et al., 2003). On the basis of these collective findings, it is conceivable that the molecular chaperones contained within epididymosomes could prevent the misfolding and aggregation of their cargo proteins in this environment of intense macromolecular crowding (Minton, 2005), and/or that they act in concert with lipid transport proteins to facilitate the transfer of these proteins onto the sperm surface. A caveat to this model is that CCTs did not feature among the proteins identified in human epididymosomes (Thimon et al., 2008), thus raising the possibility of species-specific differences in the function of this chaperone complex.

**Figure 2** Structural domains of some of the key molecular chaperone families involved in the production and functional maturation of spermatozoa. (A) CCT and HSPD1; N-terminal domain (NTD) contains the apical domain (yellow) necessary for peptide binding and recognition. The C-terminal region (CTD) contains the equatorial domain (pink) which facilitates the binding of each subunit of the complex and contains most ATP contact sites. Linking the CTD to the NTD is the intermediate domain (blue) that regulates the conformational change of the chaperone following ATP hydrolysis. (i) The eight subunits of the CCT/TRiC are arranged in dual octameric ring structures. (ii) Individual 60 kDa subunits each possess three domains, with the apical domain comprising the hydrophobic residues necessary for substrate binding. Selective substrate interactions are dependent on structural changes of each subunit that are induced by ATP hydrolysis. (B) HSP70 consists of an N-terminal nucleotide-binding domain (yellow) that accounts for the ATPase activity of the chaperone. The CTD contains a lid to enclose the central cavity (pink) of the substrate-binding domain (blue). This region is linked to the NTD by a highly conserved linker segment (LS) (black). (C) HSP90 consists of a highly conserved NTD which accounts for ATP hydrolysis (yellow). The NTD is attached to a highly charged linker hinge region (CL) (black) and two other distinct functional regions: a peptide binding domain (blue) and a carboxyl-terminal dimerization domain (EEVD motif) (pink).
Our own work has demonstrated that mature human and mouse spermatozoa harbour an intact CCT/TRiC complex that is predominantly expressed within the peri-acrosomal region of the sperm head (Dun et al., 2011; Redgrove et al., 2011). In the mouse this complex is present in testicular sperm but undergoes marked changes in subcellular localization during capacitation, including a striking increase in the surface expression of at least two CCT subunits (Dun et al., 2011). Furthermore, in the spermatozoa of both humans and mice it has been demonstrated that CCT/TRiC participates indirectly in sperm–ZP adhesion. This interaction appears to be attributed to the inability of CCT/TRiC to form a stable complex with ZP receptors, including ZPBP2 (zona pellucida-binding protein 2).

At present the mechanisms underpinning the capacitation-associated translocation of the CCT subunits to the sperm surface remains to be fully explored. Similarly, it is not certain how they interact with the putative ZP adhesion molecules. Nevertheless, it is of interest that the activity of CCT/TRiC is able to be positively modulated by interactions between the complex and caveolin-1, a constituent of specialized membrane microdomains known as membrane rafts (Doucey et al., 2006). This is in keeping with the demonstration that membrane rafts commonly regulate the functions of resident chaperones through the spatial constraint of their substrates (Elhyany et al., 2004). Additionally, it has been demonstrated that chaperones play important roles in maintaining the stability of membrane raft-associated protein complexes (Chen et al., 2005). Such findings take on added significance in light of the key role that membrane rafts play in the capacitation-associated remodelling of the sperm surface and in ZP adhesion (Nixon et al., 2009; Asano et al., 2010).

**Heat shock protein 60 (HSPD1)**

HSPD1 (formerly HSP60) is normally found in the mitochondria where it is responsible for the transportation and refolding of proteins (Cheng et al., 1989; Reading et al., 1989). The protein typically oligomerizes into a complex structure arranged as a two stacked heptameric rings (Cheng et al., 1990) with a large central cavity in which the unfolded client proteins bind via hydrophobic interactions (Fenton et al., 1994; Fig. 2A). HSPD1 has three domains: an apical domain, an equatorial domain and an intermediate domain (Ranford et al., 2000). The equatorial domain accommodates both the ATPase activity and the binding site for the additional ring (Fig. 2A). The intermediate domain undergoes a conformational change when ATP is bound that allows interactions to occur with client proteins (Ranford et al., 2000). It is well documented that HSPD1 works in conjunction with chaperonin 10 (HSP10), formerly HSP10, a co-chaperone that causes the central cavity to enlarge and enhances its ability to accommodate client proteins (Ranford et al., 2000).

HSPD1 is expressed during the early stages of human spermatogenesis where it is restricted to the spermatogonial cells that line the basal epithelium (Lachance et al., 2010). Although HSPD1 has been identified in mature human spermatozoa ([Mitchell et al., 2007; Naaby-Hansen and Herr, 2010]; see below), our knowledge of its function in spermatozoa is largely attributed to the work conducted in the mouse. In this species, HSPD1 is expressed in spermatozoa and spermatocytes, but is then apparently lost during the later stages of spermatogenesis only to be reacquired by spermatozoa as they pass through the caput epididymidis (Asquith et al., 2005). This differential pattern of HSPD1 expression bears a striking resemblance to that documented for other chaperones, including clathrin, and suggests that these proteins fulfil independent roles during different phases of sperm maturation. In the case of HSPD1, it has been shown that the epididymal secretary form of the protein resides in electron dense, amorphous structures termed dense bodies (Asquith et al., 2005; Fig. 3). Although they differ substantially from epididymosomes, the appearance of dense bodies within the precise region of the epididymis where spermatozoa acquire the capacity to recognize and bind to the ZP, implicates these structures in the functional remodelling of the sperm surface during epididymal maturation. Indeed, it has been suggested that they may mediate the bulk transfer of proteins onto the sperm surface during this process (Asquith et al., 2005; Dun et al., 2010). To date only limited proteomic analysis of these structures has been achieved, however, our data strongly suggest that in addition to HSPD1 they also contain HSPE1 and HSP90B1. Evidence from independent groups have also implicated these structures in the transfer of carbohydrate-rich aggregates that promote the formation of sperm rosettes (Monclus et al., 2007) and the bactericidal permeability-increasing protein to the acrosomal membrane (Yano et al., 2010).

HSPD1 also appears to fulfil an important function during capacitation. This was first recognized when HSPD1 was identified as a major target for capacitation-associated tyrosine phosphorylation, an event that is critical for the acquisition of the spermatozoa’s ability to interact with the oocyte (Ecroyd et al., 2003; Asquith et al., 2004). Interestingly, this modification appears to promote the expression of HSPD1 on the outer leaflet of the plasma membrane of ~25% of live capacitated spermatozoa (Asquith et al., 2004). This subpopulation of spermatozoa are believed to represent those that are capable of binding to the ZP. This is corroborated by the fact that this expression pattern is found in virtually all spermatozoa bound to the ZP but is lost once the cells have undergone acrosomal exocytosis, as would be expected of cell surface molecules involved in primary ZP interactions (Asquith et al., 2005). Nevertheless, antibodies directed against HSPD1 failed to inhibit zona binding, suggesting that the protein plays an indirect role in this process. Such findings prompted the formulation of a novel hypothesis whereby chaperone activity was implicated in the remodelling of the sperm surface architecture by promoting the assembly and/or external presentation of functional ZP-receptor complexes. Subsequent work to identify the HSPD1 client proteins in capacitated mouse spermatozoa has to date revealed that it interacts with HSPE1, CCT/TRiC and ADAMTS10 (Walsh et al., 2008; Dun et al., 2011, 2012).

Studies on human spermatozoa have also identified HSPD1 on the cell surface (Naaby-Hansen and Herr, 2010), although it is interaction with alternative proteins and their role in mediation of sperm–oocyte interactions remains to be fully elucidated in our species. Nevertheless, this chaperone is among several that have been identified in the fluids secreted from the oviduct epithelium and have been shown to interact with the human spermatozoa (Boillard et al., 2004; Lachance et al., 2007). Although a number of different hypotheses for the role of these oviductal secretions exist, one proposal is that they are essential in facilitating acquisition of the spermatozoa’s ability to interact with and fertilize the oocyte (Yang and Yanagimachi, 1989; Hunter, 1991; Kim et al., 1996; Wang et al., 1998; Slavik and Fulka, 1999; Buhi, 2002). Conversely, it has been shown that...
incubation of human spermatozoa with recombinant HSPD1 significantly inhibits the tyrosine phosphorylation of p81 (the major phosphotyrosine-containing protein of human spermatozoa) (Galantino-Homer et al., 1997). It has therefore been suggested that exogenous HSPD1 may counteract the signalling pathway that underpins capacitation-associated tyrosine phosphorylation (Lachance et al., 2007). If similar results were achieved in vivo, it would suggest that this protein may act in a manner analogous to that of a decapacitation factor (Nixon et al., 2006). HSPD1 could therefore be one of the oviductal factors that renders spermatozoa quiescent while they are stored in the Fallopian tube (Suarez and Pacey, 2006).

Heat shock protein 70 family

The 70 kDa HSPs (HSP70: HSPA1A, HSPA2/HSP70-2, HSPA4, HSPA5/BiP/GRP78, HSPA6/HSP70B, HSPA7/HSP70B, HSPA8, HSPA9, HSPA12A, HSPA12B, HSPA13, HSPA14, HSPA1B, HSPA1L, HSPA4L) are a ubiquitously expressed family of folding catalysts with a modular architecture comprising three major functional domains: a conserved N-terminal ATPase domain, a substrate-binding domain and a C-terminal domain that acts as a lid for the substrate-binding domain (Tavaaria et al., 1996; Mayer and Bukau, 2005; Fig. 2B). In the ATP bound state, the lid is held in an open position that permits the substrate-binding domain to interact with hydrophobic segments within substrate proteins. These transient interactions stimulate ATPase activity and promote the closure of the binding pocket (Mayer and Bukau, 2005). This substrate binding and release cycle is augmented by co-chaperones from the family of J-domain proteins (primarily HSP40 in eukaryotes) that target HSP70s to their substrates, and is further fine-tuned by nucleotide exchange factors, which influence the longevity of the HSP70-substrate complex (Youker and Brodsky, 2007). HSP70 function is also coupled to the action of other chaperones, such as those of the HSP90, HSP-organizing protein and HSP100 (Glover and Lindquist, 1998) families. The primary function of HSP70 rests with its ability to transiently bind to partially synthesized or denatured peptide sequences, thereby preventing their aggregation and allowing them to (re)fold into a functional state. However, by virtue of its ability to stabilize client proteins in a partially folded state, HSP70 also aids in the transmembrane transport of proteins, and in their assembly into functional complexes (Mayer and Bukau, 2005).

HSPA2

A novel, testis-specific member of the HSP70 family, known as HSPA2, has been identified in several species (Allen et al., 1988; Maekawa et al., 1989). Targeted mutation of the Hspa2 gene has revealed that the chaperone has an essential role in the transition of spermatogenic cells through the late meiotic stages of spermatogenesis (Mori et al., 1997). Specifically, it was shown that homozygous mutant Hspa2−/− mice support normal spermatogenesis until postnatal Day 15 when increasing numbers of pachytene spermatocytes become apoptotic and differentiation beyond this stage begins to falter (Allen et al., 1996; Mori et al., 1997). Spermatogenic cell
development appears to arrest in prophase of meiosis I resulting in an absence of spermatids. Detailed characterization of HSPA2 in these cells revealed two primary roles. First, HSPA2 supports the formation of a heterodimeric complex between CDC2 and cyclin B1 during the transition between G1 to S-phase and then from G2 to M-phases of meiosis (Zhu et al., 1997). Secondly, HSPA2 appears to act as a component of the synaptonemal complexes (Mori et al., 1997) and thereby assists with chromosome crossover during meiosis. More recent analyses have revealed that, after the completion of meiosis, HSPA2 acquires a new function as a chaperone of spermatid-specific DNA packaging transition proteins (Govin et al., 2006). These proteins serve as an intermediary, replacing histones before themselves being replaced by protamines during spermiogenesis (Lewis et al., 2003; Govin et al., 2004; Caron et al., 2005; Kimmins and Sassone-Corsi, 2005). Owing to its ability to escort transition proteins and mediate their assembly into DNA packaging structures, HSPA2 acts as a major regulator of genome reorganization in differentiating spermatids.

Interestingly, orthologues of HSPA2 are present in the testes of many animals, suggesting that this chaperone may play a conserved role across phyla (Eddy, 1999). Hspa2 mRNA is abundant in human testis but, unlike the mouse, it is not restricted to this tissue with additional transcripts being detected in skeletal muscle, the ovary, intestine and brain. Early reports that a HSP70 protein was expressed on the surface of human spermatozoon (Miller et al., 1992) using a monoclonal antibody that recognizes multiple HSP70 family proteins have recently been substantiated by the work of Herr and colleagues (Naaby-Hansen et al., 2010) who demonstrated that HSPA2, in addition to HSPAS5 (see below), is among the repertoire of calcium-regulated proteins expressed on the human sperm plasma membrane.

The significance of HSPA2 in mature human spermatozoa has been eluded to by the studies of Huszar et al., who have proposed that the relative levels of HSPA2 expression may be used as an objective biochemical marker of sperm maturity and hence, fertilizing potential (Huszar et al., 2000). Indeed, HSPA2 expression is significantly reduced in infertile patients whose spermatozoa possess increased cytoplasmic retention (Huszar and Vigue, 1993) resulting from a developmental defect in the last phase of spermiogenesis (Huszar and Vigue, 1990).

In studies designed to examine the stage of the fertilization process compromised by low HSPA2 expression, a substantial defect in ZP adhesion was revealed (Huszar et al., 1994, 2007). On the basis of such findings it has been proposed that human HSPA2 may play a secondary role in the remodeling of the sperm plasma membrane during spermiogenesis (Huszar et al., 2000) to facilitate the formation of the ZP-binding sites. Such a notion is in keeping with the biphasic expression of HSPA2, first in spermatocytes related to meiosis, and then at the time of terminal spermiogenesis in elongated spermatids (Govin et al., 2006; Wu et al., 2011). This proposal takes on added significance in light of our own recent work that has identified HSPA2 as a major component of several human sperm protein complexes, a subset of which is expressed on the surface of the cell and harbour known zona adhesion receptors (unpublished observations). Taken together these findings raise the intriguing prospect that, in addition to its well-characterized role in spermiogenesis, HSPA2 may also participate in the assembly of functional ZP receptor complex(es).

HSPA5

Another member of the HSP70 family that has been identified in mammalian spermatozoa is that of heat shock 70 kDa protein 5 (HSPA5, formerly GRP78/BiP). HSPA5 is commonly localized in the lumen of the endoplasmic reticulum (ER) where it plays a pivotal role in protein transport, folding and assembly (Lee, 2005). HSPA5 interacts with several stress sensors (Bertolotti et al., 2000) but disassociates from these sensors as unfolded proteins accumulate in the ER lumen. This dissociation enables HSPA5 to aid in protein folding while simultaneously promoting the activation of the released sensors and the initiation of ER-stress signalling pathways (Lee, 2005). It is therefore perhaps not surprising that Hspa5 mRNA expression is rapidly elevated in Sertoli cells following an exposure to insults such as nonylphenol (Gong et al., 2009), a widely distributed environment contaminant linked to the disruption of testicular development and decrease male fertility. However, in addition to this protective role, recent evidence suggests that HSPA5 may also play important role(s) in the function of mature human and mouse spermatozoa.

As previously mentioned (see section HSPA2), early studies reported the presence of multiple forms of HSP70 on the surface of human spermatozoa (Miller et al., 1992). Such results have been substantiated by Naaby-Hansen and Herr (2010) who were able to demonstrate that HSPA5 was among a cohort of proteins that was accessible for surface labelling with both biotin and radiiodine (Naaby-Hansen and Herr, 2010). Indeed, this study identified seven members from four different HSP families as putative residents of the human sperm surface. In addition to HSPA5, these included: HYOU1 (hypoxia up-regulated 1), HSP90AA1 (formerly, HSP86/HSPC1), HSPD1 and several isoforms of HSPA2 and HSPA1L (Naaby-Hansen and Herr, 2010). Although the HSPA2 and HSPA1L proteins are most likely to be incorporated into the plasma membrane during spermiogenesis, the origin of surface expressed HSPA5, and that of the other chaperones identified in this study, remains to be elucidated. It has been reported that strong HSPA5 expression is present in the cytoplasm of human spermatoocytes and round spermatids (Lachance et al., 2010). However, HSPA5 is also known to be abundantly expressed in the epididymal epithelium, epididymosomes (Lachance et al., 2010), seminal plasma (Pilch and Mann, 2006) and oviductal epithelium (Boilard et al., 2004; Lachance et al., 2007; Marin-Briggeler et al., 2010) raising the possibility that additional protein is transferred onto the sperm surface during their post-testicular maturation. Such interaction(s) may be mediated by the chaperone’s ATPase domain, a region that has been shown to possess an affinity for sulfogalactosyl-glycolipid (Mamelak and Lingwood, 1997, 2001; Mamelak et al., 2001), the major glycolipid component of the mammalian sperm plasma membrane.

While the role of human sperm surface HSPA5 remains to be determined, it is noteworthy that it featured among a group of calcium-binding proteins identified in an elegant 45Ca-overlay assay (Naaby-Hansen et al., 2010). It is therefore possible that it acts in concert with additional Ca2+ sensing machinery, such as calreticulin (see below), to modulate intracellular calcium concentrations ([Ca2+]i). In this context, it is noteworthy that recombinant HSPA5 is able to bind to the sperm acrosomal cap (Marin-Briggeler et al., 2010), and upon doing so is able to augment the cells response to...
progesterone-stimulus and significantly increase the \([\text{Ca}^{2+}]_i\) (Lachance et al., 2010). In an alternative study, exogenous HSPA8 was also found to influence the ability of sperm to engage in oocyte interactions in a calcium-dependent manner (Marin-Briggler et al., 2010). Indeed, the binding of recombinant HSPA5 initially had a negative impact on ZP interaction. However, this effect was abrogated, and HSPA5 was actually able to enhance sperm–ZP interaction, if the incubation media was modified through the replacement of calcium with strontium. Taken together, these findings lend support to the notion that surface expressed HSPA5 influences \([\text{Ca}^{2+}]_i\), and therefore the capacitation and fertilizing competence of human spermatozoa.

It is possible however that the protein may fulfill a different role in the spermatozoa of other species. In the mouse for instance, HSPA5 has also been identified on the extracellular surface of spermatozoa. Yet, in this species the chaperone appears to be associated with a number of additional client proteins to form a supramolecular complex (Han et al., 2011). One such protein is ADAM7 (a disintegrin and metalloprotease 7), a protease that is transferred onto the sperm surface via epididymosomes as the cells transit the epididymis (Oh et al., 2009). It has been shown that the incorporation of ADAM7 is indirectly linked to presence of two additional ADAM proteins (ADAM2 and ADAM3; Kim et al., 2006) that are known to be important for sperm migration in the female reproductive tract and adhesion to the ZP (Muro and Okabe, 2011). Two particularly interesting findings to arise from this work are that members of the HSPA5 complex, including ADAM7, reside within detergent-resistant membranes (membrane rafts) and that the assembly of this complex is promoted during sperm capacitation (Han et al., 2011). Such findings share analogy with other putative chaperone complexes (Dun et al., 2011, 2012) and lend support to the concept that the raft environment may stabilize and/or promote the formation of functional chaperone complexes during sperm activation (Dun et al., 2010).

**HSPA8**

Heat shock 70 kDa protein 8 (HSPA8, previously known as HSPA10/HSC70) is a member of the heat shock cognate (HSC) subgroup of chaperones that, unlike canonical HSPs, is constitutively expressed and performs functions related to normal cellular processes. In addition to traditional roles of protein folding, HPSA8 has been implicated in the catalysis of ATP-dependent uncoating of clathrin-coated vesicles during transport of membrane components through the cell (Gething and Sambrook, 1992), the promotion of lysosomal degradation of intracellular proteins (Chiang et al., 1989; Gething and Sambrook, 1992), and may actually have antagonistic effects to HSP70 in terms of the intracellular trafficking of newly synthesized proteins (Goldfarb et al., 2006). The participation of HSPA8 in the dynamics of clathrin-coated vesicles may be important in terms of acrosome biogenesis since this event requires the combined processes of clathrin-coated vesicle trafficking and confluence. In support of this model, HSPA8 and its putative co-chaperone rDJL, have been shown to form a stable complex with clathrin (Yang et al., 2005) and to co-localize within the developing acrosome of differentiating rat germ cells. The importance of HSPA8 in spermatogenesis is also emphasized by its association with the testis-specific serine kinase 6 (TSSK6) (Spiridonov et al., 2005). TSSK6 belongs to a group of proteins that are expressed during the late stages of spermiogenesis and is responsible for the phosphorylation of a myriad of substrates, including the histones H1, H2A, H2AX and H3 (Spiridonov et al., 2005). These phosphorylation events may be important for chromatin compaction and DNA packaging since targeted disruption of the Tssk6 gene produces a sterility phenotype in male mice accompanied by reduced sperm numbers, impaired DNA condensation and abnormal sperm morphology and motility (Spiridonov et al., 2005). Importantly, HSPA8 does not serve as a substrate for TSSK6 but rather appears to maintain the TSSK6 structure and may play a critical role in targeting the kinase to specific subcellular sites.

At present there is limited information regarding the presence or function of HSPA8 in mature human or mouse spermatozoa. However, HSPA8 is present on the surface of the oviductal epithelium and has been implicated in the binding of spermatozoa to these cells (Elliott et al., 2009). Such dynamic interactions are of significant interest as they serve to enhance sperm survival in the female reproductive tract prior to fertilization (Pollard et al., 1991; Suarez et al., 1991; Dobrinski et al., 1997; Fazieli et al., 1999; Fazieli et al., 2003; Brewis et al., 2005; Suarez and Pacey, 2006; Holt and Fazieli, 2010). In boar spermatozoa, this pro-survival property seems to be critically dependent upon HSPA8 since pretreatment of oviductal epithelium preparations with anti-HSPA8 antibody significantly negated their ability to maintain sperm viability (Elliott et al., 2009). In contrast, the enhancement of sperm survival was able to be rescued by substituting the oviductal epithelium fraction with exogenous recombinant HSPA8 (Elliott et al., 2009). This pro-survival activity of HSPA8 is conserved across the spermatozoa of several ungulate species (bovine, porcine and ovine) (Elliott et al., 2009; Lloyd et al., 2009). However, the mechanism by which HSPA8 is able to induce such responses has yet to be resolved.

**Calmegin/calserpin**

Originally identified as calcium binding, ER-resident chaperones, calmegin (CLGN) and calserpin (CALR3) are the testis-specific homologues of the ubiquitously expressed lectin chaperones, calnexin (CANX) and calreticulin (CALR), respectively. Despite extensive sequence similarities, the recruitment of unique testicular variants of CANX and CALR may be a reflection of the fact that this organ presents a special case for the control of ER protein folding because of the unique environment in which it functions. Not only do the testes experience temperatures that are typically several degrees below that of the rest of the body, but the developing germ cells require the assembly of a novel proteome and extensive remodelling of cellular organelles to produce specialized structures such as the acrosomal vesicle and the sperm flagellum (van Lith et al., 2007).

CLGN is expressed exclusively from mid-pachytene spermatocytes through to the formation of spermatids (Watanabe et al., 1992). During this key phase of the spermatogenic process, the chaperone is believed to transiently interact with the nascent glycoproteins synthesized within the ER (Ikawa et al., 1997) and destined for the acrosomal matrix and the plasma membrane of mature spermatozoa. Interestingly, coinciding with the loss of the ER during spermatogenesis, Clgn expression is arrested and the protein is not able to be detected in mature spermatozoa (Watanabe et al., 1992; Yoshinaga et al., 1999). In contrast to the situation for HSPD1 described earlier, CLGN is not reacquired during epididymal transit.
Nevertheless, despite its absence in mature spermatozoa, the chaperone has been identified as being critical for the development of the cells’ ability to engage with the oocyte during fertilization. This is evidenced by targeted disruption of the Cgln gene, which leads to the generation of male mice that are virtually sterile due to an inability to navigate beyond the uterotubal junction of the female reproductive tract in vivo and to bind to the ZP in vitro. Interestingly, a similar infertility phenotype has also been documented for Calr3 knockout mice in addition to that of mice lacking ADAM1A (Nishimura et al., 2004), ADAM2 (Cho et al., 1998), ADAM3 (Yamaguchi et al., 2006) and angiotensin-converting enzyme (Hagaman et al., 1998).

Collectively, these findings have led to the suggestion that a number of the proteins required to facilitate zona adhesion must be synthesized within the ER of developing germ cells before being folded and/or assembled through the cooperative action of CLGN and CALR3 (Ikawa et al., 1997; Muro and Okabe, 2011). Specifically, calmodulin appears to be required for the assembly of the sperm surface s-fertillin complex (heterodimer of ADAM1B and ADAM2) in addition to that of the testis-specific s-fertillin complex (heterodimer of ADAM1A and ADAM2). The latter of these complexes appears necessary for the presentation of ADAM3 on the sperm surface. In contrast, CALR3 directly associates with ADAM3 and controls its maturation (Ikawa et al., 2011), suggesting that CLGN and CALR3 have distinct and specific roles in the maturation of ADAM proteins. The fact that ADAM3 is the only protein commonly disrupted or replaced in Cgln and Calr3 knockout sperm (Yamaguchi et al., 2006; Ikawa et al., 2011; Muro and Okabe, 2011) suggests that it plays a central role in sperm migration into the oviduct in addition to sperm–ZP adhesion. Interestingly, the interaction of sperm chaperones and ADAM proteins is not restricted to those mentioned above, nor do they occur exclusively in testicular sperm cells. For instance, it has been shown that ADAM7 forms stable complexes with the molecular chaperones, CANX and HSPAS5, in sperm membranes (Han et al., 2011). Recent work from our own laboratory has also identified a related member of the ADAM superfamily, ADAMTS10 (ADAM with thrombospondin type I motifs number 10), as a putative client protein of HSPD1 (Dun et al., 2012). As indicated above, this molecular chaperone has also been implicated in the capacitation-dependent assembly and/or presentation of multimeric sperm receptor complexes. These findings raise the intriguing possibility that interaction between chaperones and ADAM proteins may be a widespread phenomenon that regulates a range of developmental processes associated with sperm maturation.

Calreticulin

Calreticulin (CALR) is a calcium binding, lectin-like, chaperone that is ubiquitously expressed within the ER of virtually all cells in higher organisms. The protein is highly conserved (Michalak et al., 1992) and divided into three domains: a globular N-terminal domain, a central proline-rich P-domain and a C-domain that binds Ca\(^{2+}\) with a relatively high capacity but low affinity (Nash et al., 1994; Krause and Michalak, 1997). Despite the presence of an N-terminal signal sequence and a C-terminal KDEL ER retention signal, a subpopulation of CALR has been localized to several different intracellular compartments (e.g. nucleus and cytoplasm) in addition to the plasma membrane of many cell types (Krause and Michalak, 1997). While it remains unclear as to how the protein is differentially localized to these regions, it is apparent that it has a myriad of different functions outside the ER environment. For instance, CALR has been implicated in the regulation of intracellular Ca\(^{2+}\) homeostasis (Fiegel et al., 1989; Michalak et al., 1992; Krause and Michalak, 1997), modulation of steroid sensitive gene transcription (Burns et al., 1994; Dedhar et al., 1994) and the regulation of integrin-mediated calcium signalling and cell adhesion (Coppolino et al., 1997; Kwon et al., 2000).

In terms of spermatozoa, CALR was first purified as a calcium-binding protein from rat spermatogenic cells (Nakamura et al., 1991) before being localized to the developing acrosomes of these cells during spermiogenesis (Nakamura et al., 1992a, b, 1993). Immunohistochemical and ultrastructural studies revealed that CALR was most abundantly expressed in the acrosome of both round spermatids and mature rat spermatozoa, with weaker labelling of other subcellular structures observed in spermatocytes, spermatids and Sertoli cells. Subsequent studies on mouse (Nakamura et al., 1992a, b), bull (Ho and Suarez, 2003) and human spermatozoa (Naaby-Hansen et al., 2010) have revealed that CALR is also expressed in a similar acrosomal location in these cells. However, additional labelling was revealed within the cytoplasmic droplet and midpiece region of human spermatozoa (Naaby-Hansen et al., 2010) and in the principal piece of the bull sperm flagellum (Ho and Suarez, 2003).

Although Calr-null mice have been generated by homologous recombination, the embryonic lethality of this mutation (Mesaì et al., 1999) has prevented a definitive assessment of the role of CALR in sperm function. Nevertheless, it has been demonstrated that mouse spermatozoa are rapidly immobilized, in a dose-dependent manner, when exposed to CALR antibodies (Nakamura et al., 1992a, b). Furthermore, CALR has been shown to strongly co-localize with the inositol 1,4,5-trisphosphate receptor (IP\(_3\)R) in the putative Ca\(^{2+}\) storage sites of the acrosome and neck of human and bull spermatozoa (Ho and Suarez, 2003; Naaby-Hansen et al., 2010). These latter findings are of significance as IP\(_3\)R has been implicated in the regulation of intracellular Ca\(^{2+}\) (Burns et al., 1998). A testis-specific homologue of PDI (PDILT) has also been localized to round spermatids and spermatocytes. The presence of CALR in the ER lumen but a subset has been detected in subcellular localizations (Turano et al., 2002; Ellerman et al., 2006; van Lith et al., 2007). The disulphide isomerase activity is achieved by virtue of the fact that PDIs contain four thioredoxin domains (CXXC) (Chivers et al., 1997): two that are redox active and an additional two that are required for either structural or substrate recognition and binding activity (Klappa et al., 1998). A testis-specific homologue of PDI (PDILT) is expressed in the seminiferous tubules and the interstitium (van Lith et al., 2007). PDILT has also been localized to round spermatids and spermatocytes.
spermatids in mice and is believed to form a multiprotein complex with CLGN in a manner analogous to that of PDI and CANX in somatic cells (van Lith et al., 2007). Functional cooperation between CLGN and PDILT enables recognition of the N-terminal region of nascent polypeptides and simultaneous trapping of free cysteines to be used as substrates to fold protein backbones into their native conformation. It is thought that PDILT may also be important for remodelling events in the ER during meiosis and that it has the potential to assist in the expulsion of excess cytoplasm during spermiogenesis (van Lith et al., 2007).

A number of PDIs have been identified in epididymal fluid and implicated in the folding of epididymal secretory glycoproteins. For instance, a substantial reduction in the abundance of PDIA6, a PDI known to bind in a redox-dependent manner to HSPAS (Mezghrani et al., 2001; Jessop et al., 2009), occurs in human spermatozoa as they transit from the corpus to the cauda epididymis (Akama et al., 2010). The apparent loss of PDIA6 might reflect the fact that this protein is required during the early phases of epididymal maturation but is subsequently shed once the cells have acquired functional competence. Conversely, the expression of PDIA3 remains unchanged in epididymal sperm (Akama et al., 2010), suggesting that its role in disulphide bond formation may be essential for the membrane remodelling events that characterize epididymal maturation and capacitation (Akama et al., 2010).

The events that underpin sperm binding and fusion with the oolemma are among the other well-documented functional roles of the PDIs (Ellerman et al., 2006). Typically these events are reliant on extensive conformational changes in the participating fusion proteins. Such changes are, in turn, often mediated by thiol–disulphide exchange(s) that is catalysed by proteins with disulphide isomerase activity (Sanders, 2000; Hogg, 2002; Matthias et al., 2002). To date, at least 4 of the 17 members of the mammalian PDI family (Elgaard and Ruddock, 2005) have been identified in spermatozoa [including: PDI, PDIA3, PDIA4 and PDIA6 (Stein et al., 2006)]. Interestingly, each of these PDIs have proteins been localized to the equatorial segment of the mature sperm cell (Ellerman et al., 2006), a region that is critical in mediation of sperm–oocyte fusion (Yanagimachi, 1994a, b). Significantly, it has been shown that pre-incubation of capacitated sperm with anti-PDIA3 antibodies significantly inhibits the formation of the multimolecular complexes that facilitate fusion between the oolemma and the sperm surface (Ellerman et al., 2006).

Heat shock protein 90

Unlike most of the other HSPs whose abundance increases upon heat stress, the HSP90 family [HSP90: HSP90AA1/HSP90α, HSP90AB1/ HSP90β, HSP90B1/endoplasmin/GP96/GRP94] is already extremely abundant accounting for between 1 and 2% of the total soluble proteins in non-stressed cells (Lai et al., 1984). HSP90AA1 and HSP90AB1 display modest differences in the C-terminal dimerization domains but both generally form homodimers. Although HSP90 is largely hydrophobic, it contains two highly charged domains: one is the hinge domain located between the N- and C-terminals and the other lies in the C-terminal domain (Fig. 2C). These domains, in concert with exposed hydrophobic surfaces, are involved in defining HSP90 substrate-binding characteristics (Binart et al., 1989; Csermely et al., 1998). HSP90 normally associates with co-chaperones such as HSP70 and promotes folding and prevention of protein aggregation (Picard, 2002). Like most chaperones, HSP90 utilizes the binding and hydrolysis of ATP via its ATPase domain to drive the opening and closing of its molecular clamp.

Owing to its ubiquitous expression it has proved difficult to definitively assess the function of HSP90 in male gametes using traditional knockout strategies. Indeed, mouse embryos lacking the Hsp90ab1 gene die at implantation (Voss et al., 2000). Nevertheless, the use of gene trap insertions has enabled investigation of the role of Hsp90aa1 in gamete formation (Grad et al., 2010) and revealed that Hsp90aa1 deficient mice are completely sterile. This defect is not attributed to a corresponding loss of Hsp90ab1, the levels of which remain unchanged in Hsp90aa1−/− mice. Similarly, the development of the reproductive system appears to be normal, but spermatogenesis is arrested at the pachytene stage of meiosis I, a phenotype that resembles that reported for Hspa2 deficient mice. This defect appears to be attributed, at least in part, to the association between HSP90AA1 and CDC2 (Grad et al., 2010). The chaperone also forms important interactions with nuclear autoantigenic sperm protein, a protein that is essential for binding and transporting the testis-specific linker histone H1T, from the cytoplasm of primary spermatocytes to the nucleus, implicating it as a functional regulator of meiosis I (O’Rand et al., 1992). In mature mouse spermatozoa, it has been shown that HSP90AA1 and HSP90B1 are both targets for capacitation-dependent tyrosine phosphorylation (Ecroyd et al., 2003; Asquith et al., 2004; Baker et al., 2006). At present, the significance of the tyrosine phosphorylation of HSP90 family chaperones during capacitation remains to be established. It is possible that it may result in conformational modifications and concomitant changes in substrate specificity, which then facilitates downstream events associated with the fertilization process. In keeping with this notion, HSP90B1, like that of HSPD1, becomes expressed on the surface of ~25% of the live capacitated sperm population (Asquith et al., 2004). As mentioned previously, this population are thought to represent the cohort of cells that is capable of interacting with the ZP. This is again corroborated by the fact that HSP90B1 is lost from spermatozoa once they have completed their acrosome reaction (Asquith et al., 2004).

Conclusions

The preceding data emphasize the notion that a suite of molecular chaperones are intimately involved in modulating the production and functional activity of mammalian spermatozoa. While the pleiotropic function of molecular chaperones in the cellular networks that characterize sperm development remains to be fully elucidated, it is apparent that their highly coordinated protein machinery fulfills a range of diverse roles (Fig. 4; Supplementary data, Table S1). In early germ cells these roles extend to controlling numerous important signalling pathways involved in cell-cycle progression, telomere maintenance, apoptosis, mitotic signal transduction, vesicle-mediated transport and targeted protein degradation (Hartl et al., 2011). Disruption of normal germ cell development can be achieved by the deactivation of a number of chaperones, resulting in azospermia possibly because of a failure to disassemble the synaptonemal complex that holds homologous chromosomes together during key stages of meiosis. Such targeted gene deletions result in a complete loss of diplotene spermatocytes, stressing their importance in cellular development (Grad et al., 2010). Furthermore, the activation of gene silenced germ cells during the later stages of spermatogenesis adds additional
importance to chaperones in the bio-chemical/physical modifications that occur post meiosis to render the immature cells functionally competent. This is no more evident than when observing the Clgn and Calr3 gene knockout studies performed by Okabe’s group. Although these testes and ER-specific chaperones are not involved in cytoskeletal protein assembly, their elimination leads to what appears morphologically functional cells that are nevertheless incapable of passing through the various regions of the female reproductive tract and interacting with the ovulated oocyte. These gene manipulation strategies provide some of the most convincing evidence that chaperones are essential in the activation of morphologically mature sperm, but important questions still remain with respect to their downstream molecular targets.

Interestingly a number of observations of the testis have been made which show that chaperones residing in early germ cells disappear from the germ line only to reappear in the segment of the epididymis where sperm gains the ability to swim and to recognize the ZP (Fig. 4; Supplementary data, Table S1). In fact, the fluids of the epididymis provide an environment in which the functions of molecular chaperones are paramount. The residency of chaperones in epididymosomes and dense bodies is suggestive of their critical role in passing essential cargo from the surrounding support cells of the epididymis and facilitating their transfer to the maturing cell.

Illuminating data have further revealed the necessary functional alterations that are made to the plasma membrane of spermatozoa in response to the physiological environment of the female reproductive tract. Not only are a number of key post-translational modifications made to chaperones but also their surface exposure, commensurate with the presentation of a number of potential ZP receptors, have enhanced our functional understanding of capacitation. Overlapping expression of many of the key chaperone classes suggests that the functional cooperation of these chaperones leads to the formation of a ‘super-chaperone complex’ on the surface of capacitated mammalian spermatozoa. Whether the assembly of such multimeric zona recognition complexes represents the sole mechanism for sperm–zona recognition or whether there is biological redundancy in this system involving the expression of alternative chaperones are paramount. The residency of chaperones in epididymosomes and dense bodies is suggestive of their critical role passing essential cargo from the surrounding support cells of the epididymis and facilitating their transfer to the maturing cell.

Figure 4 Chaperone expression during spermatogenesis and post-testicular sperm maturation. A suite of molecular chaperones have been implicated in the successive maturational phases that culminate in the production of functionally competent mammalian spermatozoa. Many of these molecular chaperones have essential pleiotropic roles in the differentiation and functional maturation of the male gamete, extending from the control of signalling pathways involved in cell-cycle progression and synaptonemal complex assembly (spermatogenesis) to the complex biochemical and biophysical modifications that occur during post-testicular sperm maturation in the male (epididymal maturation) and female (capacitation) reproductive tracts. Interestingly, a number of the chaperones that reside in early germ cells disappear only to reappear in spermatozoa following their passage through discrete regions of the epididymis. This transfer of chaperones, and their putative client proteins, to the maturing cell may be mediated by epididymosomes and/or dense bodies. Additional chaperones are also secreted by the oviductal epithelium and appear to modulate capacitation and/or elicit a pro-survival effect. Abbreviations: m, mouse; h, human; r, rat; b, bovine; p, porcine; o, ovine, e, equine.
zona-recognition molecules is still an open question. On the one hand, redundancy might be expected in such an important biological process, and on the other hand, targeted disruption of a single chaperone gene, calmodin, can induce sterility in mice via mechanisms that appear to involve the inhibition of sperm—zona recognition (Yamagata et al., 2002). Further analysis of molecular chaperones is therefore warranted as a means of providing novel targets for contraceptive intervention and as diagnostic biomarkers for male infertility.

**Supplementary data**

Supplementary data are available at http://humupd.oxfordjournals.org/.

**Authors’ roles**

M.D.D. was involved in the study design, conducting the literature review, analysis of identified data and manuscript and figure drafting; R.J.A. was involved in the study design, analysis of identified data and manuscript drafting; B.N. was involved in the study design, conducting literature the review, analysis of identified data and manuscript and figure drafting.

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**Conflict of interest**

The authors declare that there is no conflict of interest as defined by the guidelines of the International Committee of Medical Journal Editors (ICMJE; www.icmje.org).

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